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DOCKET NO.: ISIS-4502 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:
Richard H. Tullis

Confirmation No.: 9155

Application No.: 08/078,768

Group Art Unit: 1631

Filing Date: June 16, 1993

Examiner: James Martinell

For: Oligonucleotide Therapeutic Agent And Methods Of Making Same

EXPRESS MAIL LABEL NO: EL 999287071 US
DATE OF DEPOSIT: July 8, 2004

EL999287071US

MS Appeal Brief - Patent
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPEAL BRIEF TRANSMITTAL
PURSUANT TO 37 CFR § 1.192

Transmitted herewith in triplicate is the APPELLANT'S SUBSTITUTE APPEAL BRIEF in this application with respect to the Official Communication received from The United States Patent and Trademark Office on **June 8, 2004**.

☒ Also transmitted herewith:

- ☒ Response To Official Communication Dated June 8, 2004.
- ☒ Appellant's Substitute Appeal Brief Pursuant to 37 CFR §1.192.
- ☒ Courtesy copies of references cited in Substitute Appeal Brief.
- ☒ Courtesy copy of the Declaration of Dr. Sidney M. Hecht, and a courtesy copy of the Supplemental Information Disclosure Statement filed June 17, 2002.

☐ Petition is hereby made under 37 CFR § 1.136(a) (fees: 37 CFR § 1.17(a)(1)-(4) to extend the time for response to the Office Action of to and through comprising an extension of the shortened statutory period of month(s).

☒ The fee for the Appeal Brief was paid upon filing of the initial Appeal Brief, therefore no fee is due at this time. However, if this is deemed to be inaccurate, please charge any deficiency or credit any overpayment to Deposit Account No. 23-3050

☒ The Commissioner is hereby requested to grant an extension of time for the appropriate length of time, should one be necessary, in connection with this filing or any future filing submitted to the U.S. Patent and Trademark Office in the above-identified application during the pendency of this application. The Commissioner is further authorized to charge any fees related to any such extension of time to Deposit Account 23-3050. This sheet is provided in duplicate.

☐ A check in the amount of **$ .00** is attached. Please charge any deficiency or credit any overpayment to Deposit Account No. 23-3050.

☐ Please charge Deposit Account No. 23-3050 in the amount of **$ .00**. This sheet is attached in duplicate.

☒ The Commissioner is hereby requested to grant an extension of time for the appropriate length of time, should one be necessary, in connection with this filing or any future filing submitted to the U.S. Patent and Trademark Office in the above-identified application during the pendency of this application. The Commissioner is further authorized to charge any fees related to any such extension of time to deposit account 23-3050. This sheet is provided in duplicate.

Date: July 8, 2004

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Sir:

RESPONSE TO OFFICIAL COMMUNICATION DATED JUNE 8, 2004

In response to the Office communication dated **June 8, 2004**, reconsideration is respectfully requested in view of the amendments and/or remarks as indicated below:

☐ **Amendments to the Specification** begin on page of this paper.

☐ **Amendments to the Claims** are reflected in the listing of the claims which begins on page of this paper.

☐ **Amendments to the Drawings** begin on page of this paper and include an attached replacement sheet.

☒ **Remarks** begin on page 2 of this paper.

REMARKS

The appeal brief filed April 15, 2004 is alleged to be defective for presentation of evidence not previously of record. Appellant notes that reference to U.S. Patent No. 5,595,078 was a typographical error. Reference to that patent has been removed from the accompanying Substitute Appeal Brief.

Appellant also notes that the following references cited in the Communication mailed June 8, 2004 were submitted in response to statements made by the Examiner in the final rejection dated December 15, 2003 (*see, e.g.,* Office action dated December 15, 2003 at page 3) and were cited by Appellant for the Examiner's convenience to ascertain that the compounds GENASENSE and VITRAVENE fall within the scope of the present claims on appeal:

Hayes, D.F., "Bcl-2 inhibition in the treatment of cancer: clinical studies with the Bcl-2 antisense oligonucleotide G3139", in BEYOND CHEMOTHERAPY, EMERGING TARGETED THERAPIES FOR THE TREATMENT OF CANCER, Symposium Proceedings, San Francisco, California, May 11, 2001, pages 12-18;

Webb, *et al.*, Bcl-2 antisense therapy in patients with non-Hodgkin's lymphoma, THE LANCET, **1997 Apr 19**, 349(9059):1137-1141 (1997);

The Orange Book;

U.S. Patent No. 4,689,320;

U.S. Patent No. 5,264,423;

U.S. Patent No. 5,276,019;

U.S. Patent No. 5,442,049; and

U.S. Patent No. 5,595,978.

As the aforementioned references were not submitted by way of an information disclosure statement or by way of a declaration during prosecution of the application, however, Appellant has removed reference thereto in the accompanying Substitute Appeal Brief.

Similarly, Appellant notes that the following references cited in the Communication mailed June 8, 2004 were cited during prosecution by Appellant for the Examiner's convenience to ascertain, for example, that stabilized oligonucleotides were within the skill of those in the art at the time of filing:

Harvey *et al.*, *Biochemistry*, 12(2):208 (1973); and

Malkievicz *et al.*, *Czech. Chem. Comm.*, 38:2953 (1973).

See, e.g., Amendment mailed June 17, 2002 at page 7; Declaration of Dr. Stanley T. Crooke at page 5. As copies of the aforementioned references were not submitted during prosecution of the application, however, Appellant has removed reference thereto in the accompanying Substitute Appeal Brief.

Appellant clarifies by way of the Substitute Appeal Brief the location in the record of the following references:

U.S. Patent No. 3,687,808;

Fingl and Dixon, Chapter One, "General Principles", In THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, 4th edition, L.S. Goodman and A. Gilman, Eds. (1970);

Mirabelli *et al.*, *Anti-Cancer Drug Design*, 6:647-661 (1991);

Crooke, *Ann. Rev. Pharmacol. Toxicol.*, 32:329-376 (1992);

Cossum, *J. Pharm. and Exp. Ther.*, 267(3):1181-1190 (1993); and

Stepkowski *et al.*, *J. Immunol.*, 153:5336-5346 (1994).

Courtesy copies of the references are submitted with the Substitute Appeal Brief in addition to courtesy copies of the Supplemental Information Disclosure Statement filed June 17, 2002 and Declaration of Dr. Sidney M. Hecht for the Examiner's convenience.

Appellant respectfully submits that the Substitute Appeal Brief is in condition for consideration by the Examiner and response thereto. If the Examiner believes a telephone conference would expedite resolution of the issues on appeal, the undersigned may be contacted at 215-568-3100.

Respectfully submitted,

Date: July 8, 2004

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Enclosures
Substitute Appeal Brief
Courtesy copies of:

U.S. Patent No. 3,687,808
Supplemental Information Disclosure Statement filed June 17, 2002
Declaration of Dr. Sidney M. Hecht
Fingl and Dixon, Chapter One, "General Principles", In THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, 4th edition, L.S. Goodman and A. Gilman, Eds. (1970)
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Alexandria, VA 22313-1450

Sir:

APPELLANT'S SUBSTITUTE APPEAL BRIEF PURSUANT TO 37 C.F.R. § 1.192

This substitute brief is being filed in response to the Communication dated June 8, 2004 and in support of Appellant's appeal from the rejections of claims 64-76 and 78-83 dated December 15, 2003. A Notice of Appeal and Request for Oral Hearing were filed April 15, 2004.

1. REAL PARTY IN INTEREST

Based on information supplied by Appellant and to the best of the undersigned's knowledge, the real party in interest in the above-identified patent application is ISIS Pharmaceuticals, Inc., a corporation of Delaware, which is the current assignee.

2. RELATED APPEALS AND INTERFERENCES

There are no other appeals or interferences known to Appellant, Appellant's legal representative, or the assignee that will directly affect or be directly affected by or have a bearing on the Board's decision in the pending Appeal.

3. STATUS OF CLAIMS

Claims 64-76 and 78-83 are pending in this patent application and are the subject of this Appeal. Claims 64-76 and 78-83 appear in Appendix A. The claims do not stand or fall together.

4. STATUS OF AMENDMENTS

No amendments to the claims were made in response to the Final Office Action dated September 10, 2002, the finality of which was withdrawn pursuant to 37 C.F.R. § 1.129. In response to the nonfinal Office Action dated June 17, 2003, Appellant filed a Reply pursuant to 37 C.F.R. § 1.111 wherein claim 75 was amended to delete the phrase "at a temperature between 0°C and 80°C" and claim 77 was canceled. The Final Office Action dated December 15, 2003 does not reflect entry of these claim amendments. Appellant is entitled to entry and consideration of the claim amendments pursuant to 37 C.F.R. § 1.129, and they are reflected in the presentation of claims in Appendix A. No amendments to the claims have been made subsequent to the Final Office Action dated December 15, 2003. Accordingly, claims 64-76 and 78-83 as amended in the response to the Office Action dated June 17, 2003 are involved in the present Appeal.

5. SUMMARY OF INVENTION

Appellant's invention relates to the use of oligonucleotides to specifically inhibit the expression of a target protein in a cell. The invention defined by the pending claims is the product of the surprising discovery made by the Appellant more than twenty years ago that oligonucleotides may be used to regulate protein synthesis in cells via hybridization to nucleic acids. Appellant's discovery provides for the systematic design and use of oligonucleotide agents to specifically block the translation of a target nucleic acid.

In seeking patent protection for his discovery, Appellant presents independent claims 64, 73, 75, 78, and 80. The invention, as recited in independent claim 64, relates to methods for selectively inhibiting the expression of a target protein in a cell producing messenger

ribonucleic acids encoding both the target protein and other proteins without inhibiting the expression of other proteins. This can be accomplished by synthesizing an oligonucleotide having a base sequence substantially complementary to a subsequence of a messenger ribonucleic acid encoding the target protein. The oligonucleotide is introduced into the cell where hybridization of the oligonucleotide to the subsequence of the messenger ribonucleic acid occurs to inhibit the expression of the target protein.

Independent claim 73 also recites methods for selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acids encoding the target protein and other proteins without inhibiting the expression of the other proteins. According to the method of claim 73, one selects a synthetic oligonucleotide that has enhanced resistance against nuclease enzymes and a base sequence substantially complementary to a subsequence of a messenger ribonucleic acid of the cell encoding the target protein. The synthetic oligonucleotide is introduced into the cell and caused to hybridize with the messenger ribonucleic acid to inhibit the expression of the target protein.

The invention, as defined by claim 75, includes within its scope methods for selective inhibition of the expression of a target protein in a cell producing messenger ribonucleic acids encoding the target protein without inhibiting the expression of the other proteins. One selects a synthetic oligonucleotide having enhanced resistance against nuclease enzymes and a base sequence substantially complementary to a subsequence of a messenger ribonucleic acid of the cell encoding the target protein. The synthetic oligonucleotide is introduced into the cell where it hybridizes to the subsequence of messenger ribonucleic acid.

Claim 78 is directed to methods of selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acid encoding the target protein by selecting a base sequence substantially complementary to the messenger ribonucleic acid of the cell encoding the target protein, providing a synthetic oligonucleotide that is stabilized against *in vivo* degradative enzymes and having the selected base sequence, and introducing the synthetic oligonucleotide into the cell to hybridize to the subsequence of the messenger ribonucleic acid.

As defined by claim 80, Appellant's invention includes methods for selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acids encoding the target protein by selecting a plurality of base sequences that are complementary to the messenger ribonucleic acid, providing a synthetic oligonucleotide corresponding to

each of the base sequences, selecting a preferred synthetic oligonucleotide for inhibition of the target protein in a cell, and using the selected oligonucleotide to inhibit the target protein in cells.

Appellant's discovery has enabled the development of an array of therapeutic agents that address many previously unmet medical needs.

6. ISSUES

A. Whether or not the Examiner's factually unsupported surmise and speculation is adequate to refute the abundant evidence of record demonstrating that the specification and claims as originally filed enable a person of skill in the art to which the invention pertains to make and use the invention commensurate in scope with claims 64-76 and 78-83 without engaging in undue experimentation.

B. As to claim 71 alone, whether or not the Examiner has refuted the evidence of record demonstrating that claim 71 is patentably distinct over claim 1 of U.S. Patent No. 5,023,243.

7. GROUPING OF CLAIMS

Claims 64-70, 72, 80, 82, and 83 stand or fall together on appeal of issue A. Claims 71, 73-76, 78, 79, and 81 stand or fall together on appeal of issue A. Claims 64-70, 72, 82, and 83 do not stand or fall together on appeal of issue A with claims 71, 73-76, 78, 79, and 81 for the reasons set forth below.

Issue B applies only to claim 71.

8. ARGUMENT

A. The specification and claims as originally filed enable a person of skill in the art to which the invention pertains to make and use the invention without engaging in undue experimentation.

The rejection of claims 64-76 and 78-83 under 35 U.S.C. § 112, first paragraph for alleged lack of enablement is grounded in the Examiner's position that Appellant is required to have expressly taught known forms of stabilized oligonucleotides available at the time of filing in order to enable stabilized oligonucleotides other than phosphotriesters and to have successfully demonstrated inhibition of expression of a target protein using an antisense

oligonucleotide *in vivo*. Appellant respectfully disagrees with the Examiner's position. As of the priority date, one of ordinary skill in the art was aware of the existence of stabilized forms of oligonucleotides in addition to phosphotriesters and would have been guided by the disclosure of the application to the other stabilized oligonucleotides that are suitable, along with phosphotriester oligonucleotides, for *in vivo* use according to the claimed invention. Nothing more than routine experimentation was involved in determining which forms of stabilized oligonucleotides would have worked in the invention. Additionally, unmodified and modified oligonucleotides as used in the present invention are taken up by cells, are sufficiently stable to exert biological activity, and specifically hybridize to the target mRNA, as demonstrated in the examples provided in the specification. The cell culture models exemplified in the specification correlate to *in vivo* biological activity of the stabilized oligonucleotides, as substantiated by pre- and post-filing references. In short, the rejection of claims 64-76 and 78-83 under 35 U.S.C. § 112, first paragraph for alleged lack of enablement should be withdrawn because the evidence of record indicates that those skilled in the art as of October 23, 1981 having the benefit of Appellant's disclosure would have been able to practice the claimed invention without undue experimentation.

Preliminarily, Appellant respectfully asserts that claims 71, 73-76, 78, 79, and 81 are separately patentable from claims 64-70, 72, 80, 82, and 83 within the meaning of 37 C.F.R. § 1.192(c)(7). Claims 64-70, 72, 80, 82, and 83 are directed to methods of selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acids. This is done by hybridizing a synthetic oligonucleotide having a nucleotide sequence substantially complementary to the subsequence of the messenger ribonucleic acid encoding the target protein to the subsequence of the messenger ribonucleic acid to specifically inhibit expression of the target protein. Claims 71, 73-76, 78, 79, and 81 recite methods for selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acids. Expression of the target protein is inhibited by a synthetic oligonucleotide having a base sequence substantially complementary to a subsequence of the messenger ribonucleic acid wherein the synthetic oligonucleotide is stabilized to inhibit degradation by nucleases (claims 71, 78, 79, and 81) or has enhanced resistance against nuclease enzymes (claims 73-76).

Because claims 64-70, 72, 80, 82, and 83 do not require enhanced resistance to degradative enzymes or stabilization, in contrast to claims 71, 73-76, 78, 79, and 81, the

enablement rejection of claims 64-76 and 78-83 based upon an asserted "problem with uptake and stability of unmodified oligonucleotides" (Office Action mailed June 17, 2003, page 10) is limited to claims 64-70, 72, 80, 82, and 83. Therefore, claims 71, 73-76, 78, 79, and 81 are separately patentable from claims 64-70, 72, 80, 82, and 83.

The enablement requirement of 35 U.S.C. § 112, first paragraph, mandates that the specification teach those skilled in the art how to make and use the claimed invention without undue experimentation. *In re Wands*, 858 F.2d 731, 736-737, 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988) (citing *Minerals Separation, Ltd. v. Hyde*, 242 U.S. 261, 270 (1916)). The test of enablement is **not** whether **any** experimentation is necessary, but whether, if experimentation is necessary, it is **undue**. *In re Angstadt*, 537 F.2d 498, 504, 190 U.S.P.Q. 214, 219 (C.C.P.A. 1976). The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. *Wands*, 858 F.2d at 737, 8 U.S.P.Q.2d at 1404.

The factors to be considered in determining whether any necessary experimentation is undue include:

i. the breadth of the claims;
ii. the nature of the invention;
iii. the state of the prior art;
iv. the level of one of ordinary skill;
v. the level of predictability in the art;
vi. the amount of direction provided by the inventor;
vii. the existence of working examples; and
viii. the quantity of experimentation needed to make or use the invention based on the content of the disclosure.

Id. (citing *In re Forman*, 230 U.S.P.Q. 546, 547 (Bd. Pat. App. & Int. 1986)). In order to make a rejection, the examiner has the burden to establish a reasonable basis to question the enablement provided for the claimed invention. *In re Wright*, 999 F.2d 1557, 1561-62, 27 U.S.P.Q.2d 1510, 1513 (Fed. Cir. 1993). Assuming that sufficient reason for such doubt exists, a rejection for failure to teach how to make and/or use will be proper on that basis. *In re Marzocchi*, 439 F.2d 220, 223-24, 169 U.S.P.Q. 367, 370 (C.C.P.A. 1971). The burden then shifts to the Appellant to provide persuasive arguments, supported by suitable proofs

where necessary, that one skilled in the art would be able to make and use the claimed invention using the application as a guide. *In re Brandstadter*, 484 F.2d 1395, 1407, 179 U.S.P.Q. 286, 294 (C.C.P.A. 1973).

The record demonstrates that, as of the time of filing, one of ordinary skill in the art would have been able to make and use the claimed invention using the application as a guide. No undue experimentation was required. Consideration of the *Wands* factors compels a finding of enablement: (1) the breadth of the solicited claims reasonably correlates to the enabled examples using phosphotriester oligonucleotides; (2) numerous modified oligonucleotides were known to one of skill in the art at the time of filing; (3) one of skill in the art of molecular biology in 1981 was highly sophisticated; (4) the specification as fled provided ample guidance, including examples, to one of skill in the art at the time of filing as to how to make and use the invention, which is all that is required for enablement; and (5) nothing more than routine experimentation was required to determine which modified oligonucleotides are most effective in the methods of the invention. Indeed, the record is replete with evidence – facts – underscoring the enablement of the present claims. The Examiner has met these facts only with surmise and skepticism.

It has been asserted that the present invention would not work *in vivo* using double-stranded oligonucleotides. (Office Action of June 17, 2003, page 3.) However, there has been no evidence adduced whatsoever for the assertion that a double-stranded oligonucleotide would not work in the claimed methods. This is an immutable requirement for maintenance of the rejection for alleged lack of enablement. The Examiner has merely stated that the term "hybridization" refers to the formation of a double-stranded nucleic acid by annealing of two single-stranded molecules and that the instant application does not disclose the formation of triplex DNA. (Office Action mailed June 17, 2003, page 3.) Appellant has not limited his invention to single-stranded oligonucleotides. Appellant asserts that double-stranded oligonucleotides work in the claimed methods, as supported, for example, by Mercola and Cohen (*Cancer Gene Therapy*, 2(1):47-59, 48-49 (1995)). Disclosure of the mechanism by which double-stranded oligonucleotides work in the invention is not required for enablement. Appellant need only have taught how to make and use the invention without undue experimentation. This Appellant has done.

1. Enablement does not require that Appellant expressly teach known forms of stabilized oligonucleotide available at the time of filing in order to enable stabilized oligonucleotides other than phosphotriesters.

The Examiner maintains that Appellant must have taught forms of stabilized oligonucleotides other than phosphotriesters to have enabled claims 71, 73-76, 78, 79, and 81, the claims requiring stabilization. Appellant disagrees.

Section 112 requires the specification to be enabling only to persons "skilled in the art to which it pertains, or with which it is most nearly connected." *DeGeorge v. Bernier*, 768 F.2d 1318, 1323, 226 U.S.P.Q. 758 (Fed. Cir. 1985). Thus, a patent need not teach, and preferably omits, what is known in the art. *In re Buchner*, 929 F.2d 660, 661, 18 U.S.P.Q.2d 1331, 1332 (Fed. Cir. 1991); *Paperless Accounting, Inc. v. Bay Area Rapid Transit System*, 804, F.2d 859, 864, 231 U.S.P.Q. 649 (Fed. Cir. 1986) ("A patent applicant need not include in the specification that which is already known to and available to the public."); *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 U.S.P.Q. 81, 94 (Fed. Cir. 1986). It has long been the law that a person skilled in the art is deemed to possess not only basic knowledge of the particular art, but also "the knowledge of where to search out information" for section 112 purposes. *In re Howarth*, 654 F.2d 103, 105, 210 U.S.P.Q. 689 (C.C.P.A. 1981).

Nowhere in the specification are the methods of the invention limited to phosphotriester-stabilized oligonucleotides. In fact, throughout the entirety of the specification, it is clearly stated that phosphotriester oligonucleotides are simply one representative example of the stabilized oligonucleotides that may be used in the methods of the invention. For example, the specification states at page 3, "[i]n a presently preferred embodiment of the invention, by way of example and not necessarily by way of limitation, a stabilized oligonucleotide, preferably in a phosphotriester form, is provided . . . " and at page 4, "[t]he preferred oligonucleotide . . . for increased stability, may be transformed to a more stable form, such as a phosphotriester form, to inhibit degradation during use." The application again states at page 5 that the oligonucleotide "can be transformed to a more stable form, such as a phosphotriester form, to inhibit degradation. . . ." Given the language of the specification including "such as," "preferred," and variations thereof, one of ordinary skill in the art readily understands that other forms of stabilized oligonucleotides were contemplated and equally useful in the methods of the invention.

That other forms of stabilized oligonucleotides were known in the art at the time of filing has been firmly established by the declarations of eminent scientists in the field, including Dr. Jerry L. Ruth (August 29, 1994 Declaration, Part 5A; April 14, 1995 Declaration, Part A), Dr. Dennis E. Schwartz (August 19, 1994 Declaration, Part 5A; April 14, 1995 Declaration Part A), and Dr. Stanley T. Crooke (Part 5). It is a fact that stabilized oligonucleotides suitable for use in the invention were known and were available to those of ordinary skill in the art in 1981. There is no countervailing evidence of record, none whatsoever.

References of record in this application also support this fact. For example, U.S. Patent No. 3,687,808 to Merigan *et al.* describes stabilized phosphorothioate oligonucleotides available as early as 1972 (submitted by way of supplemental information disclosure statement filed June 17, 2002, a courtesy copy of which is submitted herewith). Miller *et al.* (*Biochemistry*, 13(24): 4887-4906 (1974) ("Miller 1974")) describe the stabilized alkylphosphotriester DNA analogs described in the application. Matzura and Eckstein (*Eur. J. Biochem.*, 3: 448-452 (1968)) describe the nuclease resistance of phosphorothioate oligonucleotides. Agarwal and Riftina (*Nuc. Acids Res.*, 6:9, 3009-3024 (1979)) show the synthesis of oligonucleotides containing methyl and phenylphosphonate linkages. DeClercq *et al.* (*Virology*, 42:421-428 (1970)) set forth the resistance of thiophosphate-substituted oligonucleotides to degradative enzymes. Miller *et al.* (*Biochem.*, 20(7): 1874-1880 (1981)) report a stabilized alkyl phosphonate DNA analog having activity *in vitro*. Holy ("Synthesis and Biological Activity of Some Analogues of Nucleic Acids Components," in PHOSPHORUS CHEMISTRY DIRECTED TOWARDS BIOLOGY, W.J. Stec, Ed., Pergamon Press, 53-64, 1980) describes modified nucleotide analogs having hydroxyl-containing aliphatic chains that are stable *in vivo* and display inhibitory and substrate activities.

Not only would one having ordinary skill in the art have readily understood that stabilized forms of oligonucleotides in addition to phosphotriesters were contemplated by the invention, but an artisan of ordinary skill also would have known of a number of available stabilized oligonucleotide forms as of the filing date. A routine literature search by an ordinarily skilled artisan at the time of the invention would have yielded a number of available stabilized oligonucleotides suitable for use in the invention, a sampling of which have been provided on the present record. (Crooke Declaration, Parts 3 and 5; April 14, 1995

Declaration of Dr. Schwartz, page 4; April 14, 1995 Declaration of Dr. Ruth, page 4.) As Appellant is not required to teach what is known in the art, his burden has been met.

The enablement requirement does not mandate that the Appellant have presented experiments with each of the available forms of stabilized oligonucleotides to demonstrate that they actually work in the invention. Rather, enablement requires only that Appellant have taught how to determine which stabilized oligonucleotides work in the invention without undue experimentation. Appellant has satisfied this burden by providing representative examples demonstrating his invention. One having ordinary skill in the art need only have substituted for the phosphotriester oligonucleotides of Appellant's examples other known forms of stabilized oligonucleotides to determine their efficacy in the invention. This would not have required undue experimentation on the part of an artisan of ordinary skill.

2. History has proven the naysayers of *in vivo* antisense technology to be incorrect; the present invention was complete and fully enabled in 1981.

The Examiner maintains that the methods of claim 64-76 and 78-83 would not work *in vivo* due to lack of cellular uptake, instability, and unpredictability of hybridization to the target mRNA. The Examiner has placed much reliance on the Gura (*Science*, 270: 575-577 (1995)), Rojanasakul (*Adv. Drug Delivery Revs.*, 18: 115-131 (1996)), and Hijiya (*PNAS USA*, 91:4499-4503 (1994)) articles allegedly to show that the present invention is not enabled for *in vivo* use. Appellant asserts that it is improper to base a conclusion of nonenablement upon these few references in view of their actual lack of significance and the numerous other references cited throughout the prosecution of the present application that contradict their allegations. The Declaration of Dr. Sidney M. Hecht, a premiere authority in the field of gene expression, supports this assertion.

Dr. Hecht first notes that any concerns raised by the Gura, Rojanasakul, and Hijiya references are directed to the clinical safety of *in vivo* use of antisense technology rather than at the efficacy of *in vivo* antisense methodologies. For example, Rojanasakul at page 118 queries "Can antisense work in living systems?" and responds by stating that while "there are studies which indicate the ***relative safety*** of antisense [oligonucleotides] *in vivo* . . . ***non-specific side effects*** of [antisense oligonucleotides] have also been reported in mice." Rojanasakul goes on to say that these safety concerns "do not diminish the potential use of

[antisense oligonucleotides] *in vivo*, and there are few examples of successful *in vivo* treatment in the absence of specialized delivery systems." (*Id.*) Rojanasakul continues, stating that "[c]onsidering the various obstacles that the antisense [oligonucleotides] must encounter prior to their action . . . ***the desired activity of [antisense oligonucleotides] is observed.***" (*Id.* (emphasis added)). Thus, Rojanasakul actually supports enablement of claims

Likewise, Dr. Hecht notes that Gura avers that "some experts in the field . . . argue that clinical trials have begun far too soon." (Gura at 575.) Dr. Hecht explains that such concerns regarding the clinical safety of antisense oligonucleotides were elicited by the side effects detected in some animal studies. For example, Gura describes one set of experiments in which lethality in monkeys administered a one-time, high-dose injection occurred as well as another set of experiments in which a transient decrease in two kinds of white blood cells and changes in heart rate and blood pressure resulted from the high dose administered. (*Id.* at 576.)

Similarly, the assertion that Hijiya characterizes the field of antisense as being "in its scientific infancy" is misplaced, according to Dr. Hecht. Hijiya makes clear that the unmodified and phosphorothioate-modified antisense oligonucleotides worked therein: "The experiments reported herein serve as a paradigm of [oligodeoxynucleotide]-based therapeutics for human malignancies." (Hijiya at 4503.) Hijiya reasons that, although *MYB* is an effective gene target of antisense oligonucleotides in human melanoma, "further development of the antisense strategy will be needed before the successful application of this technique ***in the clinic*** can be anticipated." (*Id.* (emphasis added).) Appellant likewise asserts that the Mercola reference describes several "signpost" studies in which a reduction in target protein was observed upon *in vivo* administration of antisense phosphorothioate-modified oligonucleotides complementary to the target genes in accordance with the presently claimed methods. (Mercola at 54-55.)

A demonstration of F.D.A. acceptable clinical safety is not required by the first paragraph of 35 U.S.C. § 112. Enablement does not require that the claimed invention satisfy the higher safety standards applied to drugs to be used in clinical trials. According to MPEP § 2107.03, "Office personnel should not impose on applicants the unnecessary burden of providing evidence from human clinical trials.... [I]t is improper for office personnel to request evidence...regarding the degree of effectiveness [in humans] (underlining in

original)." Enablement requires only that the application teach how to make and use the invention without undue experimentation. This requirement has been met: one having ordinary skill in the art would be able to make and use the invention without undue experimentation using only the application as a guide.

Moreover, no drug is free of toxic effects. Fingl and Dixon (Chapter One, "General Principles", In THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, 4th edition, L.S. Goodman and A. Gilman, Eds. (1970), courtesy copy enclosed, a copy of which is of record as Exhibit 2 to the Declaration of Dr. Sidney M. Hecht). This fact has been known for many years, as substantiated by Dr. Hecht, and is as true today as it was when first presented in this textbook. For some authors to question the clinical safety of a new drug paradigm is not surprising. If raising such questions were to bar patentability of new drugs, there would be no new drugs. Accordingly, some toxic effects of antisense therapeutics are to be expected. Some expected toxic effects, however, are not an indication that antisense therapeutics do not work *in vivo*.

Indeed, Dr. Hecht attests that any concerns voiced by Gura, Rojanasakul, and Hijiya regarding the use of antisense technology *in vivo* have been proven to be wrong. The successes achieved in the field of antisense technology have been witnessed, thereby ratifying the views of proponents of antisense at the time of the invention and silencing, indeed converting, many critics to what is clearly the correct view: antisense works *in vivo* as taught by the present application.

A number of articles that corroborate the *in vivo* success of antisense technology have been cited during prosecution of the present application. For example, Mirabelli *et al.* (*Anti-Cancer Drug Design*, 6:647-661 (1991), courtesy copy enclosed, a copy of which is of record as Exhibit 3 to the Declaration of Dr. Sidney M. Hecht) notes that antisense oligonucleotides have demonstrated activities against a broad array of targets, that "the therapeutic indexes of phosphorothioate oligonucleotides appear to be quite high," and that "certain phosphorothioates . . . are extremely well tolerated in animals." (Mirabelli at 651.) Mirabelli also provides evidence of successful *in vivo* trials of antisense oligonucleotides. (*See, e.g.*, Mirabelli at 653.)

Crooke (*Ann. Rev. Pharmacol. Toxicol.*, 32:329-376 (1992) ("Crooke 1992"), courtesy copy enclosed, a copy of which is of record as Exhibit 4 to the Declaration of Dr. Sidney M. Hecht) corroborates the *in vivo* stability of antisense oligonucleotides, noting that

nuclease activity of sera derived from different species varies, with human being the least active. (*See, e.g.*, Crooke 1992 at 337). Additionally, modified oligonucleotides enter cells at pharmacologically relevant concentrations. (*Id.* at 338-339.) *In vivo* pharmacokinetic studies reveal that antisense oligonucleotides are rapidly and broadly distributed following administration in mice, rabbits, and rats. (*Id.* at 342-343.) Toxicity studies reveal that phosphorothioate oligonucleotides, for example, have high therapeutic indices and exhibit toxicity only at concentrations far in excess of concentrations at which therapeutic activity is observed. (*Id.* at 344; 346-347.) Indeed, Dr. Crooke has attested to these facts on the present record. (Crooke Declaration, Parts 6b and 6c).

Further confirmation of the enablement of Appellant's invention is found in Cossum (*J. Pharm. and Exp. Ther.*, 267(3):1181-1190 (1993), courtesy copy enclosed, a copy of which is of record as Exhibit 5 to the Declaration of Dr. Sidney M. Hecht). That reference describes several *in vivo* studies in which phosphorothioate oligonucleotides were shown to be widely distributed following *in vivo* administration in nothing more than phosphate buffer at physiologic pH. (*See, e.g.*, Cossum at 1181-1182, 1186.) Additionally, Cossum acknowledges that the dosages at which non-antisense effects occur are significantly greater than those at which antisense effects are observed. (*Id.* at 1181.)

Stepkowski *et al.* (*J. Immunol.*, 153:5336-5346 (1994), courtesy copy enclosed, a copy of which is of record as Exhibit 6 to the Declaration of Dr. Sidney M. Hecht) demonstrates specific inhibition of intercellular adhesion molecule-1 (ICAM-1) expression by antisense molecule IP-3082, thereby promoting heart allograft survival. (Stepkowski *et al.* at 5338.) Extension of *in vitro* studies to *in vivo* analyses confirmed the correlation between the efficacy of antisense technology in a Petri dish and in a living organism.

Appellant submits that, not only has the Examiner failed to consider references which run contrary to the few references upon which he relies to assert a lack of enablement, but the Examiner also is relying upon statements that have been proven false. Courts have long and uniformly held that the making of an invention in the face of skepticism by the scientific community is a hallmark of nonobviousness. *Graham v. John Deere Co.*, 383 U.S. 1, 17-18 (1966). Were the Examiner's views on the topic of enablement to prevail, the same skepticism which provides a powerful indication of nonobviousness would simultaneously eviscerate patentability under the enablement standard. This cannot be the law.

It is the Examiner's position that the time lapse between publication of the present application in 1983 and publication of results of success by skilled artisans in an active area of research weighs heavily against enablement of claims 64-76 and 78-83. The time lapse between the effective filing date of the present application and the numerous references cited in support of enablement of claims 64-76 and 78-83, however, has no bearing on the claim that the instant application provides sufficient guidance to one of skill in the art to practice the claimed invention as early as the effective filing date of the instant application. As Dr. Hecht attests, had the pharmaceutical industry in 1981 immediately applied its existing knowledge of medicinal chemistry and pharmacology to the teachings of Appellant, it would have practiced the present invention. Various factors contributed to the lag, not the least of which included establishment, within an organization, of an internal "champion" for a new technology paradigm where the champion is willing to sponsor and defend reallocation of resources from existing programs to a new program. Additionally, once acceptance of the new paradigm is made, established pharmaceutical practice requires pharmacologists to perform substantial and numerous pre-clinical studies to determine the toxicological profile, pharmacokinetics, and pharmacodynamics of any potential drug. Thus, according to Dr. Hecht, it is not unexpected that the generation and reporting of pre-clinical and clinical studies by the pharmaceutical industry related to the efficacy of a potential drug does not immediately follow the publication of the first few positive *in vitro* results.

It has been observed by Fingl and Dixon that "[n]o drug is free of toxic effects." The authors further state, however, that "adverse effects do not arise solely because of the inherent toxicity of drugs and the limitations of the methods for early detection of this toxicity. *Many of the adverse effects could be avoided if drugs were used more carefully and more wisely.*" (*Id.* at 26 (emphasis added).) Further, "[t]he development and evaluation of new drugs in the United States is rigidly controlled by federal regulation administered by the Food and Drug Administration. A new drug may not be marketed for general clinical use until it has been subjected to thorough clinical pharmacological studies and until 'substantial evidence' of its efficacy and safety have been obtained from adequate, well-controlled clinical trials conducted by qualified investigators." (*Id.* at 29.)

Since both positive and negative results must be included in data packages submitted to regulatory agencies, pre-clinical and clinical trials are not performed haphazardly with selective omission of negative results. In other words, slapdash animal studies are not

performed for potential human therapeutic applications because all data collected is subjected to FDA scrutiny. Accordingly, every study is implemented pursuant to highly rigorous standards and carefully planned conditions. Animal tests suitable to regulatory agency submission require established animal colonies and adequate animal care facilities with appropriate veterinary oversight, the development of which is expensive and time-consuming. Accordingly, careful animal experiments do not yield large volumes of publications that appear in the literature quickly. They require systematic studies that may take years to accomplish. In other words, a significant delay in the reporting of pre-clinical or clinical results is entirely routine in the field of drug discovery and development.

Dr. Hecht also affirms that the numerous clinical investigations conducted on *in vivo* antisense methodologies underscore the belief of pharmaceutical companies and, hence, the skilled scientists that comprise them, in the efficacy of antisense technology *in vivo* as detailed by the present application. "Big" pharmaceutical companies became interested in antisense technology after the small pioneer companies confirmed its validity. For example, pioneer companies Hybridon Inc. and Isis Pharmaceuticals, Inc. were incorporated in 1989 for the purpose of developing antisense therapeutics. Gilead Sciences, Inc. formed in 1987 for the same purpose. Genta Inc. was established as a spin-off of Gen-Probe in 1988 with a business objective of developing antisense therapies initiated in Gen-Probe's diagnostic antisense studies. In the mid- and late 1990s, newcomers MethylGene Inc., Inex Pharmaceuticals Corp., and NeoPharma, to name only a few, joined the early-stage companies in exploiting the therapeutic aspects of antisense technology.

In contrast, as explained by Dr. Hecht, while contributing early-published papers regarding *in vitro* related research topics, individual academic researchers, who contribute much of the scientific literature, did not exploit and publish *in vivo* antisense technology. The reasons for this are varied. The exorbitant costs of animal studies, resulting from the necessity of numerous controls as well as the stringent regulations imposed by academic institutions and regulatory agencies, preclude most academic researchers from pursuing such studies absent industrial sponsorship. Additionally, the experiments conducted by most academicians are limited in scope by narrow, well-delineated areas of research interest. Accordingly, academic researchers do not perform isolated experiments that have no bearing on that research interest. Rather, academics are selective in choosing the focus of their experiments, limiting their experimental objectives to the particular area of research that fits

into the grand scheme of the research to which their careers are dedicated, for which they have received institutional approval to study, and for which they have been granted funding.

Dr. Hecht summarizes that antisense technology was developed by small, early stage companies having limited resources. In view of the need of such companies to conserve their limited resources and the knowledge of such companies that a single poorly planned trial yielding a negative outcome could devastate an entire business venture, the pioneer companies in the antisense field had every incentive to perform animal trials carefully and systematically. They conducted animal trials in a highly methodical manner and at timepoints dictated by scientific and business judgment to advance to that phase in the process of moving their drug candidates toward IND status. Pharmaceutical companies, including Isis Pharmaceuticals, Genta Inc., and Hybridon Inc. and their present or past large pharma partners including Novartis, Lilly, Abbott, Merck, Aventis, Amgen, Roche, and Boehringer Ingelheim, have invested huge amounts of time and money to verify the efficacy of antisense drugs in an effort to propel them through clinical phases and into the market. Given the enormous costs associated with drug development and marketing, pharmaceutical companies would not have invested so heavily in the development of antisense technologies if they believed antisense molecules would not work *in vivo*.

Indeed, clinical trials of antisense therapies have definitively established that antisense technology does work *in vivo* in accordance with the principles and guidance set forth in the present application. For example, the antisense drug fomivirsen (Vitravene™; Isis Pharmaceuticals, assignee of the present application) was approved by the FDA for the treatment of cytomegaloviral-induced retinitis in 1998. The patents covering fomivirsen (which may be found at http://www.fda.gov/cder/ob/default.htm) relate to compositions and methods for inhibiting propagation of a virus by employing an oligonucleotide hybridizable to a mRNA of the virus. In particular, one of the patents covering fomivirsen claims methods for treating cytomegaloviral (CMV) retinitis by administering an oligonucleotide complementary to CMV mRNA to the subject requiring treatment. In practice, administration of the 21-mer oligonucleotide directed to the major immediate-early transcriptional unit of CMV is accomplished simply by contacting cells with the oligonucleotide in a pharmaceutically acceptable carrier or diluent such as saline. The FDA-approved mode of administration of fomivirsen for treatment of CMV is intravitreal injection, though any method that would place the relevant cells in contact with the antisense

oligonucleotide would work. In short, fomivirsen works *in vivo* in accordance with principles of the presently claimed methods.

The Investigational New Drug Application (IND) for fomivirsen was filed with the FDA in 1993, three years prior to publication of the Rojanasakul reference. Pre-clinical data was included as part of the IND. Thus, prior to the publication of the opinions of skeptics now relied upon by the Examiner, those skilled in the art already had obtained and submitted *in vivo* data to the FDA, data supporting results contravening that opined by the skeptics. Thus, prior to publication of the negative opinions of skeptics, those skilled in the art had accomplished that which the skeptics opined would not work. In other words, those of skill in the art already were gathering *in vivo* data in support of their IND well before the publication of the opinions of antisense skeptics relied on by the Examiner.

The mere fact that a few naysayers have predicted that methods such as those claimed would not work is of no relevance to the enablement of the instant claims because there is undisputed evidence on the present record that such predictions were incorrect. Some level of skepticism as to advances in science and technology has always been raised, and probably always will. In fact, the magnitude of such skepticism is arguably proportional to the magnitude of the advance. In the final analysis, it is not relevant whether skeptics exist but, rather, whether they were right.

Here, the evidence of record clearly demonstrates that the skeptics that the Examiner has identified were *not* right. The invention as set forth in the application and as presently claimed has been proven to work in the years following the effective filing date of the present application. Indeed, clinical trials of antisense therapies have established that antisense technology does work in accordance with the principles and guidance set forth in the present application, thereby dispelling the criticism of antisense skeptics including Gura and Rojanasakul.

In short, no further disclosure other than that made by Appellant in 1981 was necessary for those skilled in the art to practice the inventions as presently claimed without undue experimentation. This is the hallmark of enablement, and in no way is rebutted by the mere fact that there were those who doubted whether the underlying technology would ultimately be found to work. The methods that Appellant disclosed in 1981 have been demonstrated to work repeatedly thereafter. No clearer case of satisfaction of the enablement requirement of 35 U.S.C. § 112 can be shown.

B. There is no obviousness-type double patenting.

Claim 71 stands rejected under the judicially created doctrine of obviousness-type double patenting as being allegedly unpatentable over claim 1 of U.S. Patent No. 5,023,243. Appellant traverses.

In determining whether a nonstatutory basis exists for a double patenting rejection, the issue is whether any claim in the application defines an invention that is merely an obvious variation of an invention claimed in the patent. When the claimed subject matter is patentably distinct from the subject matter claimed in a commonly owned patent, a double patenting rejection is improper. *Eli Lilly & Co. v. Barr Labs.*, Inc., 58 U.S.P.Q.2d 1865 (Fed. Cir. 2001). Any analysis employed in an obviousness-type double patenting rejection parallels the guidelines for analysis of a 35 U.S.C. § 103 obviousness determination (*In re Braat*, 19 U.S.P.Q.2d 1289 (Fed. Cir. 1991)); however, a double patenting rejection must rely on a comparison of only the claims. MPEP § 804, part III.

Claim 71 recites a method for selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acids, the method comprising the steps of synthesizing an oligonucleotide having a base sequence substantially complementary to a subsequence of a messenger ribonucleic acid, wherein the subsequence encodes the target protein, introducing the stabilized oligonucleotide into the cell, and hybridizing the stabilized oligonucleotide to the subsequence of messenger ribonucleic acid to inhibit expression of the target protein.

In contrast, claim 1 of U.S. Patent No. 5,023,243 recites a method of selectively inhibiting *in vivo* synthesis of one or more specific targeted proteins comprising the steps of synthesizing an oligodeoxyribonucleotide having a nucleotide sequence substantially complementary to at least a portion of the base sequence of messenger ribonucleic acid coding for the targeted protein. In the claims of the '243 patent, at least a portion of the oligodeoxyribonucleotide is in the form of a phosphotriester to limit degradation *in vivo*. Claim 1 of the '243 patent calls for introducing the oligonucleotide into the cell and hybridizing the oligonucleotide to the subsequence of messenger ribonucleic acid to substantially block translation of the base sequence and to inhibit synthesis of the targeted protein.

It is asserted that claim 1 of the '243 patent is a specific embodiment of claim 71, thereby rendering present claim 71 obvious. Appellant disagrees. The oligonucleotide of present claim 71 has a sequence substantially complementary to the *coding* portion of the target mRNA, whereas the sequence of the oligodeoxyribonucleotide of claim 1 of the '243 patent is substantially complementary to any region of the mRNA coding for the targeted protein. Because the portion of the target protein to which the oligonucleotide of claim 1 of the '243 patent is not limited to the coding region of the mRNA, that claim is not a specific embodiment of, and thus does not render obvious, claim 71 of the present application. No *prima facie* case of nonstatutory obviousness-type double patenting exists.

9. APPENDIX

A listing of the claims involved in the present Appeal, as amended by the response filed September 17, 2003, is provided in Appendix A.

Respectfully submitted,

Date: July 8, 2004

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Enclosures

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: **Richard H. Tullis** Confirmation No.: **9155**
Serial No.: **08/078,768** Group Art Unit: 1631
Filing Date: **June 16, 1993** Examiner: **James Martinell**
For: **Oligonucleotide Therapeutic Agent And Methods Of Making Same**

EXPRESS MAIL LABEL NO.: EL 999287071 US
DATE OF DEPOSIT: July 8, 2004

Mail Stop Appeal-Brief Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

APPENDIX A TO APPELLANT'S SUBSTITUTE BRIEF

The following constitutes a complete listing of the claims on appeal. The claims do not stand or fall together. The Amendments of September 17, 2003 in response to the Office Action of June 17, 2003 are included.

64. A method of selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acids encoding the target protein and other proteins without inhibiting the expression of the other proteins, said method comprising the steps of:

(a) synthesizing an oligonucleotide having a base sequence substantially complementary to a subsequence of a messenger ribonucleic acid said subsequence coding for the target protein,

(b) introducing the oligonucleotide into the cell; and,

(c) hybridizing the oligonucleotide to the subsequence of the messenger ribonucleic acid to inhibit the expression of the target protein.

65. The method of claim 64 wherein the entire sequence of the oligonucleotide is complementary to the subsequence of a messenger ribonucleic acid coding for the target protein.

66. The method of claim 64 wherein the oligonucleotide is at least 14 bases in length.

67. The method of claim 64 wherein the oligonucleotide is about 23 bases in length.

68. The method of claim 64 wherein the oligonucleotide is between 14 and 23 bases in length.

69. The method of claim 64 wherein the messenger ribonucleic acid is viral.

70. The method of claim 64 wherein the messenger ribonucleic acid encodes a hormone.

71. The method of claim 64 wherein the oligonucleotide is stabilized to inhibit degradation by nucleases.

72. The method of claim 64 wherein the oligonucleotide is an oligodeoxynucleotide.

73. A method of selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acids encoding the target protein and other proteins without inhibiting the expression of the other proteins, said method comprising the steps of:

selecting a synthetic oligonucleotide that has enhanced resistance against nuclease enzymes and has a base sequence substantially complementary to a subsequence of a messenger ribonucleic acid of said cell, said subsequence coding for the target protein,

introducing said synthetic oligonucleotide into the cell, and

hybridizing said synthetic oligonucleotide to the subsequence of the messenger ribonucleic acid to inhibit the expression of the target protein.

74. The method of claim 73 wherein said synthetic oligonucleotide is between 14 and about 23 bases in length.

75. **(Previously presented in Amendment submitted September 17, 2003)** A method of selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acids encoding the target protein and other proteins without inhibiting the expression of the other proteins, said method comprising the steps of:

selecting a synthetic oligonucleotide that has enhanced resistance against nuclease enzymes and has a base sequence substantially complementary to a subsequence of a messenger ribonucleic acid of said cell, said subsequence coding for the target protein, and

introducing said synthetic oligonucleotide into the cell to hybridize said synthetic oligonucleotide to the subsequence of the messenger ribonucleic acid.

76. The method of claim 75 wherein said synthetic oligonucleotide is between 14 and about 23 bases in length.

77. **(Canceled in Amendment submitted September 17, 2003)**

78. A method of selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acid encoding the target protein, said method comprising the steps of:

selecting a base sequence substantially complementary to said messenger ribonucleic acid of said cell coding for the target protein,

providing a synthetic oligonucleotide that is stabilized against *in vivo* degradative enzymes, said synthetic oligonucleotide having said selected base sequence, and

introducing said synthetic oligonucleotide into the cell whereby said synthetic stabilized oligonucleotide hybridizes to the subsequence of the messenger ribonucleic acid.

79. The method of claim 78 wherein said synthetic oligonucleotide is between 14 and about 23 bases in length.

80. A method of selectively inhibiting the expression of a target protein in a cell producing messenger ribonucleic acids encoding the target protein, said method comprising the steps of:

selecting a plurality of base sequences that are complementary to said messenger ribonucleic acid,

providing a synthetic oligonucleotide corresponding to each of said base sequences,

selecting a preferred one of said synthetic oligonucleotides for inhibition of the expression of said target protein in a cell, and

using said selected oligonucleotide for inhibition of said target protein in cells.

81. The method of claim 80 wherein said synthetic oligonucleotides are oligonucleotides stabilized against *in vivo* degradative enzymes.

82. The method of claim 80 wherein said selected synthetic oligonucleotide is between 14 and about 23 bases in length.

83. The method of claim 80 further comprising the step of synthesizing bulk amounts of said selected oligonucleotide for inhibition of said target protein *in vivo*.

DOCKET NO.: PMB9658 (ISIS-4502) PATENT

DECLARATION ACCOMPANYING REPLY
FILED UNDER EXPEDITED PROCEDURE
PURSUANT TO 37 C.F.R. § 1.129

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Tullis **Confirmation No.: 9155**

Serial No.: 08/078,768 **Group Art Unit: 1631**

Filing Date: June 16, 1993 **Examiner: J. Martinell**

For: Oligonucleotide Therapeutic Agent And Methods Of Making Same

EXPRESS MAIL LABEL NO: EV 160093356 US

Box AF
Assistant Commissioner for Patents
Washington DC 20231

Sir:

DECLARATION OF DR. SIDNEY M. HECHT PURSUANT TO 37 CFR § 1.132

I, Dr. Sidney M. Hecht, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my knowledge are true and statements made on information or belief are believed to be true. The Exhibits attached hereto are incorporated herein by reference.

2. I am the J.W. Mallet Professor of Chemistry and Professor of Biology at the University of Virginia. I serve as Chairman of the Scientific Advisory Board of Orchid BioSciences, as a member of the Scientific Advisory Boards of Xenogen, Galileo Laboratories and Palumed, and as a consultant for Isis Pharmaceuticals. I am President of Pinnacle Pharmaceuticals and a member of the Board of Directors. I also am a member of the Board of Directors of Orchid BioSciences. I serve as an Associate Editor of the *Journal of the American Chemical Society* and sit on the Editorial Advisory Boards of *Anti-Cancer Drug Design*, *Bioconjugate Chemistry* and *Current Medicinal Chemistry-Anticancer Agents*.

From 1981 to 1987 I held concurrent appointments at Smith Kline & French Laboratories, first as Vice President Preclinical R&D, then as Vice President Chemical R&D. I have been an Alfred P. Sloan Fellow and a John Simon Guggenheim Fellow at the Max Planck Institut für Experimentelle Medizin at Göttingen. In 1991 I served as a Professor Associé at the Muséum National d'Histoire Naturelle in Paris and Gastprofessor at the Eidgenössische Technische Hochschule in Zürich; I studied at the Museum again for six months during 2000. I have held numerous lectureships at other universities. I received the 1996 Cope Scholar Award of the American Chemical Society and was selected as Virginia's Outstanding Scientist for 1996. More recently I received the 1998 Research Achievement Award of the American Society of Pharmacognosy.

A copy of my curriculum vitae is attached hereto as Exhibit 1.

3. As early as 1969, I studied mechanisms of protein synthesis via gene expression and regulation thereof. As early as 1972, I co-authored scientific journal articles regarding these studies. Further I have studied the chemistry and biochemistry of nucleic acids since 1966.

My decades of experience as a biological chemist have instilled in me a knowledge of mechanisms of expression of specific genes.

4. I have read and am familiar with the contents of the above-referenced patent application. I have read and agree with the declarations of Dr. Jerry L. Ruth, Dr. Dennis H. Schwartz, and Dr. Stanley T. Crooke previously submitted in connection with the present application. I further understand that the nature of the rejection at issue in the pending application is that the Examiner believes that the pending claims are overbroad in view of the skepticism of critics of antisense technology between October 1981 and the present. It is asserted by the Examiner that the time lapse between October 1981 and the dates of publication of the numerous references cited by the Applicant to support his claim that the methods of the invention work as set forth in the application weighs heavily against the assertion that the instant application provides sufficient guidance to one of skill in the art to practice the claimed invention as early as October 1981. The purpose of this declaration is to address this issue.

I will explain that the concerns of the Examiner stemming from a review of articles by antisense critics including Gura, Rojanasakul, and Hijiya are directed to the immediate clinical applicability of antisense applications rather than to the efficacy of *in vivo* antisense technology applications and, in any event, that those concerns have been proven baseless. I will explain why a significant delay in the reporting of clinical results is routine in the field of drug discovery and development. I also will explain that the numerous clinical investigations conducted on *in vivo* antisense methodologies demonstrate the confidence of pharmaceutical companies and, hence, those skilled scientists who comprise them in the use of antisense technology *in vivo* as detailed by the present application.

In short, the opinions of naysayers that have been lodged against the validity of antisense technology were wrong when made and have been proven to be wrong. Antisense technology works *in vivo* in accordance with the principles of the present invention. The invention as set forth in the application and as presently claimed has been proven to work time and again in the years following the effective filing date of the present application. No further disclosure than that made by Applicant in 1981 was necessary to practice the invention as presently claimed. Applicant was absolutely correct in 1981 and has been proven correct repeatedly thereafter. Additionally, a significant delay in the reporting of clinical results is routine in the field of drug discovery and development, particularly in the present case, where antisense technology was developed by small pioneer companies. The large investments by pharmaceutical companies in the development of antisense technologies underscores their belief in the efficacy of *in vivo* antisense applications. Their continued investments and positive results prove the continued vitality of that belief.

5. Any concerns raised by Gura (*Science*, 270: 575-577 (1995)), Rojanasakul (*Adv. Drug Delivery Revs.*, 18: 115-131 (1996)), and Hijiya (*PNAS USA*, 91:4499-4503 (1994)) are directed toward the immediate clinical applicability of *in vivo* use of antisense technology. These authors do not question the efficacy of antisense applications *in vivo*. For example, Rojanasakul at page 118 queries "Can antisense work in living systems?" and responds by stating that while "there are studies which indicate the ***relative safety*** of antisense [oligonucleotides] *in vivo* . . . ***non-specific side effects*** of [antisense oligonucleotides] have also been reported in mice." Rojanasakul goes on to say that these safety concerns "do not diminish the potential use of [antisense oligonucleotides] *in vivo*, and there are few examples of successful *in vivo* treatment in the absence of specialized delivery systems." *Id.*

Rojanasakul continues, stating that "[c]onsidering the various obstacles that the antisense [oligonucleotides] must encounter prior to their action . . . ***the desired activity of [antisense oligonucleotides] is observed.***" *Id.* (emphasis added).

Gura, a non-research-performing reporter, avers that "some experts in the field . . . argue that clinical trials have begun far too soon." Gura at 575. Such concerns regarding the clinical safety of antisense oligonucleotides were elicited by the side effects detected in some animal studies. For example, Gura describes one set of experiments in which lethality in monkeys administered a one-time, high-dose injection occurred as well as another set of experiments in which a transient decrease in two kinds of white blood cells and changes in heart rate and blood pressure resulted from the high dose administered. *See id.* at 576.

Similarly, the assertion that Hijiya characterizes the field of antisense as being "in its scientific infancy" is misplaced. Hijiya makes clear that antisense oligonucleotides worked therein: "The experiments reported herein serve as a paradigm of [oligodeoxynucleotide]-based therapeutics for human malignancies." Hijiya at 4503. Hijiya reasons that, although *MYB* (a gene) is an effective target of antisense oligonucleotides in human melanoma, "further development of the antisense strategy will be needed before the successful application of this technique ***in the clinic*** can be anticipated." *Id.*

"No drug is free of toxic effects." *See* Fingl and Dixon (Chapter One, "General Principles", In THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, 4th edition, L.S. Goodman and A. Gilman, Eds. (1970)) (Exhibit 2). This fact has been known for many years and is as true today as it was when first presented in this textbook. For some authors, to question the clinical safety of a new drug paradigm is not surprising. If raising such questions were to bar patentability of new drugs, there would be no new drugs. Accordingly, some toxic effects of

antisense therapeutics are to be expected. Some expected toxic effects, however, are not an indication that antisense therapeutics do not work *in vivo*.

Moreover, any concerns voiced by Gura, Rojanasakul, and Hijiya regarding the use of antisense technology *in vivo* have been proven to be wrong. The successes achieved in the field of antisense technology have been witnessed, thereby ratifying the views of proponents of antisense at the time of the invention and silencing, indeed converting, many critics to what is clearly the correct view: antisense works *in vivo* as taught by the present application.

A number of articles that corroborate the *in vivo* success of antisense technology have been cited during prosecution of the present application. Further submitted with the accompanying reply is Mirabelli et al. (*Anti-Cancer Drug Design*, 6:647-661 (1991)) (Exhibit 3) which notes that antisense oligonucleotides have demonstrated activities against a broad array of targets, that "the therapeutic indexes of phosphorothioate oligonucleotides appear to be quite high," and that "certain phosphorothioates . . . are extremely well tolerated in animals." Mirabelli at 651. Mirabelli also provides evidence of successful *in vivo* trials of antisense oligonucleotides. *See, e.g.*, Mirabelli at 653.

Crooke (Annu. Rev. Pharmcol. Toxicol., 1992, 32:329-76) (Exhibit 4) corroborates the *in vivo* stability of antisense oligonucleotides, noting that nuclease activity of sera derived from different species varies, with human being the least active. *See, e.g.*, Crooke 1992 at 337. Additionally, modified oligonucleotides enter cells at pharmacologically relevant concentrations. *See id.* at 338-339. *In vivo* pharmacokinetic studies reveal that antisense oligonucleotides are rapidly and broadly distributed following administration in mice, rabbits, and rats. *See id.* at 342-343. Toxicity studies reveal that phosphorothioate oligonucleotides,

for example, have high therapeutic indices and exhibit toxicity only at concentrations far in excess of concentrations at which therapeutic activity is observed. *See id.* at 344; 346-347.

Cossum (*J. Pharm. and Exp. Ther.*, 267(3):1181-1190 (1993)) (Exhibit 5) describes several *in vivo* studies in which phosphorothioate oligonucleotides were shown to be widely distributed following *in vivo* administration in nothing more than phosphate buffer at physiologic pH. *See, e.g.*, Cossum at 1181-1182, 1186. Additionally, Cossum acknowledges that the dosages at which non-antisense effects occur are significantly greater than those at which antisense effects are observed. *See id.* at 1181.

Stepkowski et al. (*J. Immunol.*, 153:5336-5346 (1994)) (Exhibit 6) demonstrates specific inhibition of intercellular adhesion molecule-1 (ICAM-1) expression by antisense molecule IP-3082, thereby promoting heart allograft survival. *See* Stepkowski et al. at 5338. Extension of *in vitro* studies to *in vivo* analyses confirmed the correlation between the efficacy of antisense technology in a Petri dish and in a living organism.

Indeed, a search of the art of "antisense" in the PubMed database reveals approximately 16,986 references demonstrating the extensive interest of the scientific community in the technology of the presently claimed invention (Exhibit 7).

6. The Examiner asserts that the time lapse between the effective filing date of the present application and the numerous references cited in support of enablement of the solicited claims weighs heavily against the claim that the instant application provides sufficient guidance to one of skill in the art to practice the claimed invention as early as the effective filing date of the instant application. I disagree. Had the pharmaceutical industry in 1981 immediately applied its existing knowledge of medicinal chemistry and pharmacology to the teachings of Applicant, I believe that it would have practiced the present invention.

Various factors contributed to this lag, not the least of which include establishment, within an organization, of an internal "champion" for a new technology paradigm where the champion is willing to sponsor and defend reallocation of resources from existing programs to new a program. Also, once acceptance of the new paradigm is made, established pharmaceutical practice requires pharmacologists to perform substantial and numerous pre-clinical studies to determine the toxicological profile, pharmacokinetics, and pharmacodynamics of any potential drug. Thus, my extensive experience as a biological and medicinal chemist have taught me that it is not unexpected that the generation and reporting of pre-clinical and clinical studies by the pharmaceutical industry related to the efficacy of a potential drug does not immediately follow the publication of the first few positive *in vitro* results.

It has been observed by Fingl and Dixon (*see supra*, paragraph 5) that "[n]o drug is free of toxic effects." They further state, however, that "adverse effects do not arise solely because of the inherent toxicity of drugs and the limitations of the methods for early detection of this toxicity. *Many of the adverse effects could be avoided if drugs were used more carefully and more wisely.*" *Id.* at 26 (emphasis added). Further, "[t]he development and evaluation of new drugs in the United States is rigidly controlled by federal regulation administered by the Food and Drug Administration. A new drug may not be marketed for general clinical use until it has been subjected to thorough clinical pharmacological studies and until 'substantial evidence' of its efficacy and safety have been obtained from adequate, well-controlled clinical trials conducted by qualified investigators." *Id.* at 29.

Since both positive and negative results must be included in data packages submitted to regulatory agencies, clinical trials are not performed haphazardly with selective omission of negative results. In other words, slapdash animal studies are not conducted for potential

human therapeutic applications because all data collected is subjected to FDA scrutiny. Accordingly, every study is implemented pursuant to highly rigorous standards and carefully planned conditions. Animal tests suitable to regulatory agency submission require established animal colonies and adequate animal care facilities with appropriate veterinary oversight, the development of which is expensive and time-consuming. Accordingly, careful animal experiments do not yield large volumes of publications that appear in the literature quickly. They require systematic studies that may take years to accomplish. In other words, a significant delay in the reporting of pre-clinical or clinical results is entirely routine in the field of drug discovery and development.

While contributing early-published papers related to *in vitro* related research topics, individual academic researchers, who contribute much of the scientific literature, did not exploit and publish, *in vivo* antisense technology. The reasons for this are varied. The very substantial costs of animal studies, resulting from the necessity of numerous controls as well as the stringent regulations imposed by academic institutions and regulatory agencies, preclude most academic researchers from pursuing such studies absent industrial sponsorship. Additionally, the experiments conducted by most academicians are limited in scope to their existing, well-delineated areas of research interest. Accordingly, academic researchers do not perform isolated experiments that have no bearing on that research interest. Rather, academics are selective in choosing the focus of their experiments, limiting their experimental objectives to the particular area of research that fits into the grand scheme of the research to which their careers are dedicated, for which they have received institutional approval to study, and for which they have been granted funding.

7. The numerous clinical investigations conducted and patents sought on *in vivo* antisense methodologies demonstrate the confidence of pharmaceutical companies and, hence, the skilled scientists that comprise them in the use of antisense technology *in vivo* as detailed by the present application. "Big" pharmaceutical companies became interested in antisense technology after the small pioneer companies confirmed its validity. For example, pioneer companies Hybridon Inc. and Isis Pharmaceuticals, Inc. were incorporated in 1989 for the purpose of developing antisense therapeutics. Gilead Sciences, Inc. formed in 1987 for the same purpose. Genta Inc. was established as a spin-off of Gen-Probe in 1988 with a business objective of developing antisense therapies initiated in Gen-Probe's diagnostic antisense studies. In the mid- and late 1990s, newcomers MethylGene Inc., Inex Pharmaceuticals Corp., and NeoPharma, to name only a few, joined the early-stage companies in exploiting the therapeutic aspects of antisense technology.

In short, antisense technology was developed by small, early stage companies having limited resources. In view of the need of such companies to conserve their limited resources and the knowledge of such companies that a single poorly planned trial yielding a negative outcome could devastate an entire business venture, the pioneer companies in the antisense field had every incentive to perform animal trials carefully and systematically. They conducted animal trials in a highly methodical manner and at timepoints dictated by scientific and business judgment to advance to that phase in the process of moving their drug candidates toward IND status. Pharmaceutical companies including Isis Pharmaceuticals, Genta Inc. and Hybridon Inc. and their present or past large pharma partners including Novartis, Lilly, Abbott, Merck, Aventis, Amgen, Roche and Boehringer Ingelheim have invested huge amounts of time and money to verify the efficacy of antisense drugs in an

effort to propel them through clinical phases and into the market. Given the enormous costs associated with drug development and marketing, pharmaceutical companies would not have invested so heavily in the development of antisense technologies if they believed antisense molecules would not work *in vivo*.

8. In summary, the opinions of naysayers that have been lodged against the validity of antisense technology were wrong when made and have been proven to be wrong. Antisense technology works *in vivo* in accordance with the principles of the present invention. The invention as set forth in the application and as presently claimed has been proven to work time and again in the years following the effective filing date of the present application. No further disclosure than that made by Applicant in 1981 was necessary to practice the invention as presently claimed. Applicant was absolutely correct in 1981 and has been proven correct repeatedly thereafter. Additionally, a significant delay in the reporting of clinical results is routine in the field of drug discovery and development, particularly in the present case, where antisense technology was developed by small pioneer companies. The large investments by pharmaceutical companies in the development of antisense technologies underscores their belief in the efficacy of *in vivo* antisense applications. Their continued investments and positive results prove the continued vitality of that belief.

Date: 03/04/03

Dr. Sidney M. Hecht

Attachment
Exhibits 1, 2, 3, 4, 5, 6 and 7

DOCKET NO.: ISIS-4502 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Richard H. Tullis

Serial No.: 08/078,768 **Group Art Unit: 1804**

Filing Date: June 16, 1993 **Examiner: J. Martinell**

For: OLIGONUCLEOTIDE THERAPEUTIC AGENT AND METHODS OF MAKING SAME

DATE OF DEPOSIT: June 17 2002
I HEREBY CERTIFY THAT THIS PAPER IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE AS FIRST CLASS MAIL, POSTAGE PREPAID ON THE DATE INDICATED ABOVE AND IS ADDRESSED TO THE ASSISTANT COMMISSIONER FOR PATENTS, WASHINGTON, DC 20231.

TYPED NAME: John W. Caldwell
REGISTRATION NO.: 28,937

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Pursuant to 37 C.F.R. §1.56 and in accordance with 37 C.F.R. §§1.97-1.98, information relating to the above-identified application is hereby disclosed. Inclusion of information in this statement is not to be construed as an admission that this information is material as that term is defined in 37 C.F.R. §1.56(b).

☐ In accordance with §1.97(b), since this Information Disclosure Statement is being filed either within three months of the filing date of the above-identified application, within three months of the date of entry into the national stage of the above identified application as set forth in §1.491, before the mailing date of a first Office Action on the merits of the above-identified application, or before the mailing date of a first office action after the filing of request for continued

examination under §1.114, no additional fee is required.

☐ In accordance with §1.129(a), this Information Disclosure Statement is being filed in connection with ☐the first or ☐second After Final Submission, therefore:

☐ Certification in Accordance with §1.97(e) is attached; or

☐ The fee of **$180.00** as set forth in §1.17(p) is attached.

☒ In accordance with §1.97(c), this Information Disclosure Statement is being filed after the period set forth in §1.97(b) above but before the mailing date of either a Final Action under §1.113 or a Notice of Allowance under §1.311, or before an action that otherwise closes prosecution in the application, therefore:

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☐ In accordance with §1.97(d), this Information Disclosure Statement is being filed after the mailing date of either a Final Action under §1.113 or a Notice of Allowance under §1.311 but before, or simultaneously with, the payment of the Issue Fee, therefore included are: Certification in Accordance with §1.97(e); and the submission fee of **$180.00** as set forth in §1.17(p).

☒ Copies of each of the references listed on the attached Form PTO-1449 are enclosed herewith.

☐ Copies of references listed on the attached Form PTO-1449 are enclosed herewith EXCEPT THAT:

☐ In view of the voluminous nature of references **[list as appropriate]**, and the likelihood that these references are available to the Examiner, copies are not enclosed herewith.

☐ In accordance with §1.98(d), copies of the following references listed on the attached Form PTO-1449 are not enclosed herewith because they were previously cited by or submitted to the U.S. Patent and Trademark Office in patent application(s) for which a claim for priority under 35 U.S.C.§120 have been made in the instant application:

☐ Copies of references **[list as appropriate]** listed on the attached Form PTO-1449 were previously cited by or submitted to the Patent and Trademark Office in prior application Serial No. , filed .

☐ If any of the foregoing publications are not available to the Examiner, Applicant will endeavor to supply copies at the Examiner's request.

Please charge any deficiency or credit any overpayment to Deposit Account No. 23-3050. This form is submitted in duplicate.

There are no listed references which are not in the English language.

Date: June 17, 2002

John W. Caldwell
Registration No. 28,937

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Form PTO-1449 Modified List of Patent and Publications Cited by Applicant (Use several sheets if necessary) U.S. Department of Commerce Patent and Trademark Office	Docket No. **ISIS-4502**	Serial No. **08/078,768**
	Applicant **Richard H. Tullis**	
	Filing Date **June 16, 1993**	Group **1804**

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

	1	Agarwal, K.L., et al., "Synthesis and enzymatic properties of deoxyribooligonucleotides containing methyl and phenylphosphonate linkages," *Nucleic Acids Research*, July 11, **1979**, *6(9)*, 3009-3024
	2	De Clercq, E., et al., "The antiviral activity of thiophosphate-substituted polyribonucleotides in vitro and in vivo," *Virology*, **1970**, *42*, 421-428
	3	Holy, A., "Synthesis and biological activity of some analogues of nucleic acids components," *International Union of Pure and Applied Chemistry*, September 25-28, **1979**, Phosphorus Chemistry Directed Towards Biology, 53-64
	4	Matzura, H., et al., "A polyribonucleotide containing alternating → P = O and → P = S linkages," *European J. Biochem.*, **1908**, *3*, 448-452

EXAMINER	**DATE CONSIDERED**

Form PTO-1449 Modified List of Patent and Publications Cited by Applicant (Use several sheets if necessary) U.S. Department of Commerce Patent and Trademark Office	Docket No. **ISIS-4502**	Serial No. **08/078,768**
	Applicant **Richard H. Tullis**	
	Filing Date **June 16, 1993**	Group **1804**

U. S. PATENT DOCUMENTS

Examiner Initial		Document No.	Date	Name	Class	Subclass
	5	3,687,808	08/29/72	Merigan, et al.	195	28 N

FOREIGN PATENT DOCUMENTS

Examiner Initial		Document No.	Date	Country	Translation YES	Translation NO

EXAMINER	**DATE CONSIDERED**

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
08/078,768	06/16/1993	RICHARD H. TULLIS	PMB9658	9155

32650 7590 06/08/2004

WOODCOCK WASHBURN LLP
ONE LIBERTY PLACE - 46TH FLOOR
PHILADELPHIA, PA 19103

EXAMINER	
ART UNIT	PAPER NUMBER

DATE MAILED: 06/08/2004

1515-4502

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APPLICATION NO./ CONTROL NO.	FILING DATE	FIRST NAMED INVENTOR / PATENT IN REEXAMINATION	ATTORNEY DOCKET NO.

EXAMINER	
ART UNIT	PAPER
	504

DATE MAILED:

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Commissioner for Patents

The appeal brief filed April 15, 2004is defective in that it presents as evidence two references not previously of record (*viz.* Hayes, in *Beyond Chemotherapy, Emerging Targeted Therapies for the Treatment of Cancer, Symposium Proceeding*, San Francisco, CA, May 11, 2001, pages 12-18 and Webb et al, Lancet 349 (9059), 1137 (1997)). In addition, the following articles and patents are referred to in the brief, but none of these articles or patents is of record an no copy of any of these articles or patents is in the record:

(a) U.S. Patent No. 3,687,808,

(b) Harvey et al, Biochemistry 12(2), 208 (1973),

(c) Malkievicz et al, Czec. Chem. Comm. 38: 2953 (1973),

(d) Fingl et al, Chapter One, "General Principles", in *The Pharmacological Basis of Therapeutics*, 4th edition, L.S. Goodman et al (eds.) 1970,

(e) Mirabelli et al, Anticancer Drug Design 6: 647 (1991),

(f) Crooke, Annu. Rev. Pharmacol. Toxicol. 32: 329 (1992),

(g) Cossum, J. Pharm. Exp. Ther. 267(3), 1181 (1993),

(h) Stepkowski et al, J. Immunology 153: 5336 (1994),

(i) U.S. Patent No. 4,689,320,

(j) U.S. Patent No. 5,264,423,

(k) U.S. Patent No. 5,276,019,

(l) U.S. Patent No. 5,442,049,

(m) U.S. Patent No. 5,595,978,

(n) "The Orange Book", and

(o) U.S. Patent No. 5,595,078.

Entry of new evidence in an application on appeal is not a matter of right (MPEP 1207) and must be submitted as a paper separate from the appeal brief. In addition, any amendment, affidavit, or other new evidence on appeal is governed by 37 CFR §§ 1.116 and 1.195 (MPEP 1207). Since none of these conditions has been complied with, the appeal brief is defective.

Appellant is given **ONE (1) MONTH or THIRTY (30) DAYS** from the mailing date of this notice, whichever is longer, within which to supply the omission or correction in order to avoid abandonment. EXTENSIONS OF THIS TIME PERIOD MAY BE GRANTED UNDER 37 CFR 1.136(a).

Any inquiry concerning this communication or earlier communications from the examiner should be directed to James Martinell whose telephone number is (571) 272-0719. The fax phone number for Examiner Martinell's desktop workstation is (571) 273-0719. The examiner works a flexible schedule and can be reached by phone and voice mail. Alternatively, a request for a return telephone call may be e-mailed to james.martinell@uspto.gov. Since e-mail communications may not be secure, it is suggested that information in such requests be limited to name, phone number, and the best time to return the call.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Michael Woodward, can be reached on (571) 272-0722.

PLEASE NOTE THE NEW FAX NUMBER

The fax phone number for the organization where this application or proceeding is assigned is (703) 872-9306.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is (571) 272-1600.

James Martinell, Ph.D.
Primary Examiner
Art Unit 1631
6/5/07

The Pharmacological Basis of Therapeutics

FOURTH EDITION

A TEXTBOOK OF
PHARMACOLOGY, TOXICOLOGY, AND
THERAPEUTICS FOR
PHYSICIANS AND MEDICAL STUDENTS

THE MACMILLAN COMPANY
COLLIER-MACMILLAN LIMITED *London*
COLLIER-MACMILLAN CANADA LIMITED *Toronto*

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In this textbook, reference to proprietary names of drugs is ordinarily made only in chapter sections dealing with preparations. Such names are given in SMALL-CAP TYPE, usually immediately following the official or nonproprietary titles. Proprietary names of drugs also appear in the Index.

SECTION

I

Introduction

CHAPTER

1 GENERAL PRINCIPLES

Edward Fingl and Dixon M. Woodbury

The basic pharmacological concepts summarized in this chapter apply to the characterization, evaluation, and comparison of all drugs. A clear understanding of these principles will facilitate subsequent study of the individual drugs. Many of these topics have been more extensively discussed in the textbook by Goldstein and coworkers (1968) and in the symposium edited by Tedeschi and Tedeschi (1968).

SCOPE OF PHARMACOLOGY

In its entirety, *pharmacology* embraces the knowledge of the history, source, physical and chemical properties, compounding, biochemical and physiological effects, mechanisms of action, absorption, distribution, biotransformation and excretion, and therapeutic and other uses of drugs. Since a *drug* is broadly defined as any chemical agent that affects living processes, the subject of pharmacology is obviously quite extensive.

For the physician and the medical student, however, the scope of pharmacology is less expansive than indicated by the above definitions. The clinician is interested primarily in drugs that are useful in the prevention, diagnosis, and treatment of human disease, or in the prevention of pregnancy. His study of the pharmacology of these drugs can be reasonably limited to those aspects that provide the basis for their rational clinical use. Secondarily, the physician is also concerned with chemical agents that are not used in therapy but are commonly responsible for household poisoning as well as environmental pollution. His study of these substances is justifiably restricted to the general principles of prevention, recognition, and treatment of such toxicity or pollution. Finally, all physicians feel a sense of responsibility toward the growing sociological problem of the abuse of drugs.

A brief consideration of its major subject areas will further clarify how the study of pharmacology is best approached from the standpoint of the specific requirements and interests of the medical student and practitioner. At one time, it was essential for the physician to have a broad botanical knowledge, since most drugs used in therapy were obtained from plants, and since the physician had to select the proper plants from which to prepare his own crude medicinal preparations. However, relatively few drugs are still obtained from natural sources, and most of these are highly purified or standardized and differ little from synthetic chemicals. Hence, the interests of the modern clinician in *pharmacognosy* are correspondingly limited. Nevertheless, scientific curiosity should stimulate the physician to learn something of the *sources* of drugs, and this knowledge often proves practically useful as well as interesting. He will find the *history* of drugs of similar value.

The preparing, compounding, and dispensing of medicines at one time lay within the province of the physician, but this work is now delegated almost completely to the pharmacist. However, to write intelligent prescription orders, the physician must have some knowledge of the *physical and chemical properties* of drugs and their available

dosage forms, and he must have a basic familiarity with the *practice of pharmacy*. When the physician shirks his responsibility in this regard, he invariably fails to translate his knowledge of pharmacology and medicine into prescription orders and medication best suited for the individual patient. The few details essential to the writing of correct prescription orders are summarized in the Appendix.

The study of the biochemical and physiological *effects* of drugs and their *mechanisms of action* is termed *pharmacodynamics*. It is an experimental medical science that dates back only to the latter half of the nineteenth century. As a border science, pharmacodynamics borrows freely from both the subject matter and the experimental technics of physiology, biochemistry, microbiology, and pathology. It is unique mainly in that attention is focused on the characteristics of drugs. As the name implies, the subject is a dynamic one. The student who attempts merely to memorize the pharmacodynamic properties of drugs is foregoing one of the best opportunities for correlating the entire field of preclinical medicine. For example, the actions and effects of the saluretic agents can be fully understood only in terms of the basic principles of renal physiology and of the pathogenesis of edema. Conversely, no greater insight into normal and abnormal renal physiology can be gained than by the study of the pharmacodynamics of the saluretic agents.

Pharmacodynamics also deals with the *absorption, distribution, biotransformation,* and *excretion* of drugs. These factors, coupled with dosage, determine the concentration of a drug at its sites of action and, hence, both the intensity and the time course of its effects. Many basic principles of biochemistry and enzymology and the physical and chemical principles that govern the active and passive transfer and the distribution of substances across biological membranes are readily applied to the understanding of this important aspect of pharmacodynamics.

Another ramification of pharmacodynamics is the correlation of the actions and effects of drugs with their chemical structures. Such *structure-activity relationships* are an integral link in the analysis of drug action, and exploitation of these relationships among established therapeutic agents has often led to the development of better drugs. However, the correlation of biological activity with chemical structure is usually of interest to the physician only when it provides the basis for summarizing other pharmacological information.

The physician is understandably interested mainly in the effects of drugs in man. This emphasis on *human pharmacology* is justified, since the effects of drugs are often characterized by significant interspecies variation, and since they may be further modified by disease. In addition, some drug effects, such as those on mood and behavior, can be adequately studied only in man. However, the pharmacological evaluation of drugs in man may be limited for technical and ethical reasons, and the choice of drugs must be based in part on their pharmacological evaluation in animals. Consequently, some knowledge of *animal pharmacology* and *comparative pharmacology* is helpful in deciding the extent to which claims for a drug based upon studies in animals can be reasonably extrapolated to man.

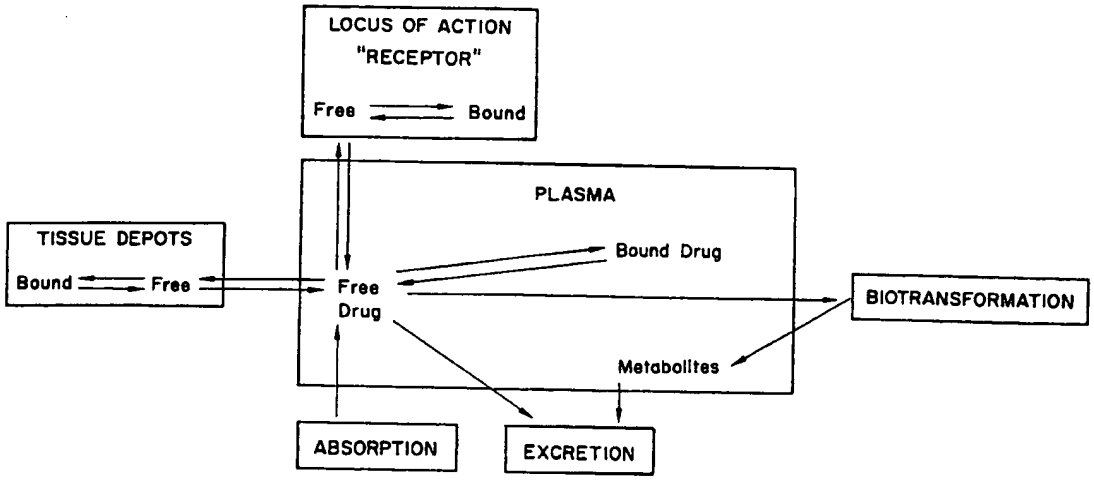
Pharmacotherapeutics deals with the *use* of drugs in the prevention and treatment of disease. Many drugs stimulate or depress biochemical or physiological function in man in a sufficiently reproducible manner to provide relief of symptoms or, ideally, favorably to alter the course of disease. Drugs of this type are designated *pharmacodynamic agents*. Other drugs, known as *chemotherapeutic agents*, are useful in therapy because they have only minimal effects on man but can destroy or eliminate parasites. Whether a drug is useful for therapy is crucially dependent upon its ability to produce its desired effects with only tolerable undesired effects. Thus, from the standpoint of the physician interested in the therapeutic uses of a drug, the *selectivity* of its effects is one of its most important characteristics.

In the medical curriculum, pharmacotherapeutics is often neglected in the initial teaching of pharmacology, because the formal course is usually taught during the preclinical years, and it is thought that the student lacks the necessary clinical background. This is unfortunate, since drug therapy is rationally based upon the correlation of the actions and effects of drugs with the physiological, biochemical, and microbiological aspects of disease. Pharmacodynamics provides one of the best opportunities for this correlation during the study of both the basic and the clinical medical sciences.

Toxicology is that aspect of pharmacology that deals with the adverse effects of drugs. It is concerned not only with drugs used in therapy but also with the many other chemicals that may be responsible for household, environmental, or industrial intoxication. The toxic effects of the pharmacodynamic and chemotherapeutic agents are properly considered an integral part of their total pharmacology. The toxic effects of other chemicals is such an extensive subject that the physician must usually confine his attention to the general principles applicable to the prevention, recognition, and treatment of drug poisonings of any cause.

ABSORPTION, DISTRIBUTION, BIOTRANSFORMATION, AND EXCRETION

To produce its characteristic effects, a drug must achieve adequate concentrations at its sites of action. Although obviously a function of the amount of drug administered, the concentrations attained also depend upon the extent and rate of its absorption, distribution, binding or localization in tissues, inactivation, and excretion. These factors are depicted in the following scheme:



PHYSICOCHEMICAL FACTORS IN TRANSFER OF DRUGS ACROSS MEMBRANES

Either directly or indirectly, the absorption, distribution, biotransformation, and excretion of a drug all involve its passage across cell membranes. It is essential, therefore, to consider the mechanisms by which drugs cross membranes and the physicochemical properties of molecules and membranes that influence this transfer. Important characteristics of a drug are its molecular size and shape; degree of dissociation or ionization of an acid, base, or salt; and relative lipid solubility of its charged and uncharged forms. Single cells and cell nuclei have a simple plasma membrane. In contrast, the intestinal epithelial boundary is represented by a single cell layer, and the skin barrier is composed of several layers of cells. Despite these anatomical differences, the diffusion and transport of drugs across these various boundaries are remarkably similar.

Cell Membranes. The classical observations by Overton and by Collander and Bärlund led to the theory that the cell (plasma) membrane consists of a thin layer of lipoid material interspersed with minute water-filled channels, often called pores. Subsequent studies have indicated that the plasma membrane consists of a bimolecular lipoid sheet bound on both sides by protein. The thickness of the membranes is of the order of 100 Å. On the basis of electron micrographic studies, two categories of membranes have been distinguished (Sjöstrand, 1967). The first type, exemplified by the plasma membrane, primarily controls the composition of the medium bounded by the membrane, by means of characteristic permeability and transport properties. The second type, exemplified by the mitochondrial membrane, is primarily associated with enzymatic function; the structure of such membranes is more complex and consists of a thicker inner surface thought to contain highly ordered multienzyme systems in the form of globular proteins.

Passive Processes. Drugs move across membranes either by passive transfer processes or by specialized active transfer systems. The membrane is not involved in the passive processes, and the drug molecules penetrate either by passage through aqueous channels in the membrane or by dissolving in the membrane substance. Both nonpolar lipid-soluble compounds and polar substances that possess sufficient lipid solubility move across the predominantly lipoid plasma membrane by *passive diffusion*. Their transfer is directly proportional to the concentration gradient across the membrane and the lipid:water partition coefficient of the drug. The greater the partition coefficient, the higher is the concentration of drug in the membrane and the faster is its diffusion. However, after a steady state is attained, the concentration of the drug is the same on both sides of the membrane. Passage through channels is called *filtration*, since it involves bulk flow of water as a result of a hydrostatic or osmotic difference across the membrane. The bulk flow of water carries with it any water-soluble molecule that is small enough to pass through the channels. Filtration is a common mechanism for transfer of many small, water-soluble, polar and nonpolar substances. The size of the membrane channels differs in the various body membranes. Capillary endothelial cells have large channels (40 Å), and molecules as large as albumin pass from the plasma to the extracellular fluid or from the plasma into the glomerular filtrate. In contrast, the channels in the red-cell membrane, the intestinal epithelium, and most cell

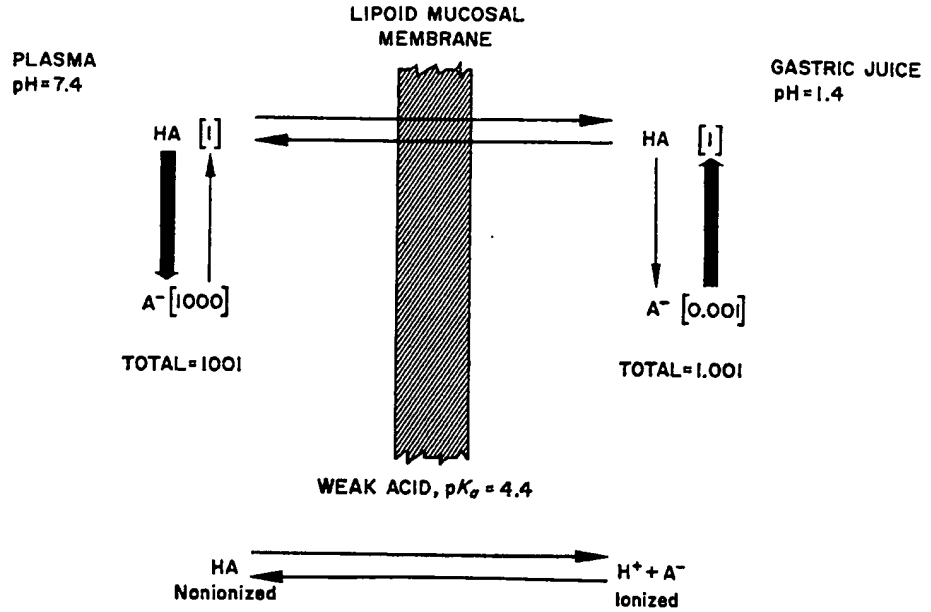


Figure 1–1. *Influence of pH on the distribution of a weak acid between plasma and gastric juice, separated by a lipoid membrane.*

Only the nonionized moiety can readily penetrate the membrane; hence, at equilibrium its concentration is the same in both compartments. The degree of dissociation of the acid on each side depends on the pH of the plasma and gastric juice. The total concentration difference between the two sides is a direct function of the pH gradient across the membrane.

The values in brackets represent relative concentrations of the ionized and nonionized forms on each side of the membrane. The thick vertical arrows point in the direction of the predominant form of the weak acid at the indicated pH.

membranes are about 4 Å in diameter and permit passage only of water, urea, and other small, water-soluble molecules. Such substances generally do not pass through channels in cell membranes if their molecular weights are greater than 100 to 200.

Most inorganic ions are sufficiently small to penetrate the pores in membranes, but their concentration gradient across the cell membrane is generally determined by the transmembrane potential (*e.g.*, chloride ion) or by active transport (*e.g.*, sodium and potassium ions).

Weak Electrolytes and Influence of pH. Most drugs are weak acids or bases and are present in solution as both the nonionized and ionized species. The nonionized portion is usually lipid soluble and can readily diffuse across the cell membrane. In contrast, the ionized fraction is often unable to penetrate the lipoid membrane because of its low lipid solubility, or to traverse the membrane channels because of its size. If the ionized portion of a weak electrolyte can pass through the channels, or through the membrane, it will distribute according to the transmembrane potential. For example, inorganic ions, like chloride, bicarbonate, and bromide, and the ionized form of drugs, like salicylate or 5,5-dimethyl-2,4-oxazolidinedione (DMO), are distributed unequally across the red-blood-cell membrane.

If the ionized portion of a weak electrolyte cannot penetrate the membrane, its distribution will be determined by its pK_a and the pH gradient across the membrane. If a pH gradient exists, the degree of ionization of the electrolyte on the two sides of the membrane will differ. Accordingly, even though the concentration of the nonionized fraction on the two sides is the same, the total concentration of the drug (ionized plus nonionized) on the two sides will also differ. To illustrate the effect of pH on distribution of drugs, the partitioning of a weak acid ($pK_a = 4.4$) between plasma (pH = 7.4) and gastric juice (pH = 1.4) is depicted in Figure 1–1. It is assumed that the gastric mucosal membrane behaves as a simple lipoid barrier that is permeable only to the lipid-soluble, nondissociated form of the acid. The ratio of nonionized to ionized drug at each pH can be calculated from the Henderson-Hasselbalch equation. Thus, in plasma, the ratio of nonionized to ionized drug is 1:1000; in gastric juice, the ratio is 1:0.001. The total concentration ratio between the plasma and the gastric juice sides of the barrier is therefore 1000:1. For a weak base with a pK_a of 4.4, the ratio is reversed. (For more detailed discussions of drug transfer by passive processes and the effect of pK_a and pH on drug movements, *see* Albert, 1952; Brodie and Hogben, 1957; Schanker, 1962.)

Specialized Active Transport Processes. The

passive processes described above do not explain the passage of all drugs across cell membranes. Specialized active transport processes appear to be responsible for the rapid cellular transfer of certain foreign organic ions and polar molecules, as well as many natural substrates such as sugars, amino acids, and pyrimidines.

Active transport processes differ from passive processes in that they exhibit selectivity, saturability, and a requirement for energy. Specialized active transport is generally thought to be mediated by carriers, membrane components that form a complex with the substance to be transported. The complex is presumed to be formed on one side of the membrane and to diffuse to the other side where the substance is released, after which the carrier returns to the original surface to repeat the process. If the process transports a substance against an electrochemical gradient (uphill transport) and is blocked by metabolic inhibitors, it is called *active transport;* ions such as Na^+, K^+, and I^-, amino acids, certain strong organic acids and bases, and ionized forms of weak electrolytes are transported across the renal tubule, choroid plexus, and liver cells in this manner. Carrier transport that shows selectivity, saturability, and blockade by metabolic inhibitors but in which the substance does not move against a concentration gradient is called *facilitated diffusion.* Glucose, pyrimidines, and some of their antimetabolites are transported across gastrointestinal epithelium and other cell membranes by this process. Transport is facilitated by attachment to a carrier and is more rapid than simple diffusion.

ABSORPTION

It is of practical importance to know the manner in which drugs are absorbed. The rate of absorption largely determines the latent period between administration and onset of action; it is also important in determining dosage. In many instances, absorption influences the choice of the route by which a drug is administered.

Many variables, in addition to the physicochemical factors discussed above, influence the absorption of drugs. Absorption from all sites of administration is dependent upon drug *solubility.* Drugs given in solution are more rapidly absorbed than those given in solid form. For those given in solid form, the rate of dissolution becomes the limiting factor in their absorption. Local conditions at the site of absorption alter solubility. Thus, at the low pH of the gastric juice, many acidic drugs are absorbed slowly because they precipitate in the fluids of the stomach, and dissolution occurs very slowly. Highly insoluble substances, such as barium sulfate, are not absorbed from the alimentary tract at all. The *concentration* of a drug also influences its rate of absorption. Drugs ingested or injected in solutions of high concentration are absorbed more rapidly than are drugs in solutions of low concentration. The *circulation to the site of absorption* also affects drug absorption is most importantly determined area, brought about by massage or local application of heat, enhances absorption of a drug, whereas decreased blood flow, produced by vasoconstrictors, shock, or other factors, slows absorption. The area of the *absorbing surface* to which a drug is exposed is one of the more important determinants of the rate of drug absorption. Obviously, drugs are absorbed very rapidly from large surface areas such as the pulmonary endothelium, peritoneal cavity, and intestinal mucosa, and factors that increase the area over which a drug is spread enhance absorption. Since the absorbing surface is determined largely by the *route of administration,* it is evident that the rate of drug absorption is most importantly determined by this factor. Often there is a choice in the route by which a therapeutic agent may be given, and a knowledge of the advantages and disadvantages of the different methods of administration is then of primary importance.

Alimentary Tract. The oral route is the most ancient method of drug administration. It is also the safest, most convenient, and most economical. Drugs given by mouth may be retained there and absorbed through the oral mucosa, or they may be swallowed and absorbed from the stomach and intestine. Disadvantages to the oral route of ingestion of drugs include emesis as a result of irritation to the gastrointestinal mucosa, destruction of some drugs by the digestive enzymes, formation with food of complexes that cannot be absorbed, and necessity for cooperation on the part of the patient.

Oral Mucosa. The mucosal lining of the mouth behaves as a lipoid barrier to the passage of drugs, and their absorption through the oral mucosa involves the same principles outlined above for most epithelial membranes. Absorption from the oral mucosa is rapid. A higher concentration of the drug in the blood may be achieved than by

absorption lower in the alimentary tract because metabolism of drugs as a result of passage through the liver is avoided, and because the drug is not subjected to possible destruction by the gastrointestinal secretions or to formation of complexes with foods. However, substances that are distasteful or that are irritating should not be given by this route. The sublingual route of administration permits rapid absorption of a variety of drugs. It is a convenient method that is often overlooked.

Gastrointestinal Tract. Absorption of drugs from the gastrointestinal tract is for the most part explainable in terms of pH-dependent, simple nonionic diffusion across the gastrointestinal epithelial membrane, as described above and depicted in Figure 1–1. For example, alcohol, a lipid-soluble nonelectrolyte, is rapidly absorbed into the blood stream by diffusion across the gastric mucosa. Weak acids, such as salicylates and barbiturates, which are predominantly nonionized in the acid gastric contents, are also readily absorbed from the stomach. In contrast, weak bases, such as morphine, quinine, ephedrine, and tolazoline, which are predominantly ionized at the pH of gastric juice, are poorly absorbed through the gastric mucosa and are absorbed mainly through the intestinal mucosa. If the gastric contents are made alkaline, acidic compounds become more ionized and are less well absorbed. Conversely, basic compounds become less ionized and are better absorbed. The pH at the surface of the intestinal epithelium where absorption occurs is about 5.3; consequently, weak bases are more readily absorbed and weak acids less readily absorbed than in the stomach. Increasing the pH of the intestinal contents enhances this difference. Quaternary ammonium compounds, streptomycin, and other completely ionized, lipid-insoluble drugs are very slowly absorbed. Succinylsulfathiazole and other drugs, even the nonionic forms of which are lipid insoluble, are also poorly absorbed from the gastrointestinal tract. The quaternary ammonium compounds may be absorbed by formation of a phosphatido-peptide intermediate.

Theoretically, the concentration ratio of a weak [illegible] between gastric juice and plasma can be as high as 1 millionfold. Experimentally, the value never exceeds 40-fold, because gastric mucosal blood flow limits the rate at which the drug can be supplied to, or removed from, the gastric juice and equilibrium is never attained. The establishment of concentration gradients of weak electrolytes across the gastric mucosa is a purely physical process and does not require an active transport system. All that is necessary is to have a membrane preferentially permeable to the nonionizable form of the weak electrolyte and the establishment of a pH gradient across the membrane. The establishment of the pH gradient is, however, an active transport process requiring expenditure of energy to secrete hydrogen and chloride ions.

Factors other than the rate of passage of drugs across the gastrointestinal mucosa influence the absorption of drugs from the alimentary tract. One of these factors is the rate of *gastric emptying*. Changes in gastric emptying might be expected to have opposite effects on absorption of weak acids and weak bases because of the difference in gastric and intestinal pH. However, because of the large surface area of the intestine, the absorption of most drugs is reduced if gastric emptying is retarded. Other factors that affect absorption, such as *solubility* of a drug in gastrointestinal fluids and *concentration* of the administered solution, have been noted above. Absorption from the alimentary tract may also be retarded or decreased if the ingested drug is unstable in gastrointestinal fluid, or if it becomes bound to food or other gastrointestinal contents. Simultaneous ingestion of food also delays absorption by decreasing gastric emptying.

The rate of absorption of solid forms of drugs is dependent mainly on their disolution rate in gastrointestinal fluids, and this factor is the basis for the oral *prolonged-action* or *sustained-release* pharmaceutical preparations. These preparations include tablets-within-tablets, slowly disintegrating tablets, pellets in capsules, slowly dissolving salts in solid or liquid suspension, and drugs embedded in ion-exchange or inert plastic matrices, all of which are designed to produce slow, uniform absorption of the drug and thereby provide a sustained effect for 8 hours or longer. In addition, they are intended to provide rapid onset of action by immediate release of sufficient active ingredient to raise the level of the drug in the blood to that producing the desired therapeutic effect. Potential advantages of such preparations are reduction in the frequency of administration of the drug as compared with conventional forms, maintenance of a therapeutic effect overnight, and decreased incidence of undesired effects by elimination of the

peaks in drug concentration that often occur after repeated administration of nonsustained-release dosage forms. Sustained-release preparations may also reduce the hazard of defaulting from prescribed treatment by allowing the patient to take the drug less often. In the case of some drugs, such as certain amphetamines, antihistamines, antitussives, and tranquilizers, uniformly effective sustained-release preparations have been achieved and are being used successfully in therapy. However, each drug must be evaluated separately for its suitability as a sustained-release preparation. It is obvious that drugs given for a brief therapeutic effect should not be in the sustained-release form. In addition, not all marketed preparations are reliable. Dissolution rate of some preparations in gastrointestinal fluid may be quite irregular because of technical problems associated with their manufacture and of variations in gastrointestinal pH, gastric emptying, intestinal motility, and other physiological factors that influence drug absorption. Since the total dose of drug ingested at one time is equal to 3 to 4 doses of the conventional form of the drug, release of the entire amount at once due to occasional failure of the material to hold together may occur and lead to toxicity. Also, failure of adequate release may compromise the therapeutic effect. Therefore, it is incumbent on the physician who uses preparations of this type to establish a need for a prolonged-action preparation and also to evaluate its uniformity, reliability, and safety. This is especially necessary since the same drugs made into sustained-release formulations by different processes or by different manufacturers may have release rates and durations of actions that vary considerably from each other. The use of these nonuniform and poorly reliable preparations cannot be justified at the present time.

Rectal Mucosa. Drugs can also be given by the *rectal* route. This is often useful when the oral route is precluded by vomiting or when the patient is unconscious. In addition, the absorbed drug does not pass through the liver before entry into the systemic circulation. However, absorption by this route is often irregular and incomplete, and many drugs cause irritation of the rectal mucosa.

Injection. The injection of medicinals has at times certain distinct advantages over oral administration. In some instances, this route of administration is essential for the drug to be absorbed in active form. Absorption is usually more rapid and more predictable than when a drug is given by mouth. The effective dose can therefore be more accurately selected. In emergency therapy, parenteral administration is particularly serviceable. If a patient is unconscious, uncooperative, or unable to retain anything given by mouth, parenteral therapy may become a necessity. On the other hand, there are several disadvantages to the injection of drugs. Strict asepsis must be maintained in order to avoid infection, an intravascular injection may occur when it is not intended, pain may accompany the injection, and it is often difficult for a patient to perform the injection himself if self-medication is a necessary procedure. Parenteral therapy is also more expensive and less safe than oral medication. Absorption of lipid-soluble drugs from subcutaneous and intramuscular sites occurs by simple nonionic diffusion through the capillary membranes into the blood and is directly proportional to the lipid:water partition coefficient of the drug. The rate of absorption is also influenced by the total area of the absorbing capillary membranes and by the solubility of the substance in the interstitial fluid. Lipid-insoluble drugs are absorbed into the blood by penetration through the relatively large aqueous pores in the endothelial membrane; larger molecules, such as proteins, gain access to the circulation by way of lymphatic channels. Some large molecules and microcrystalline substances are absorbed from these sites by phagocytosis.

Subcutaneous. Injection at this site is often utilized for the administration of medicinals. It can be used only for drugs that are not irritating to tissue; otherwise, a slough may occur. The rate of absorption following subcutaneous injection of a drug is often sufficiently even and slow to provide a fairly sustained effect. Moreover, it may be willfully varied by well-known technics. The subcutaneous injection of a suspension of a drug in a vehicle in which it is insoluble results in a very slow rate of absorption. For example, the rate of absorption of a suspension of insoluble protamine insulin is slow as compared with that of soluble insulin. The incorporation of a vasoconstrictor agent in a solution of a drug to be injected subcutaneously also retards absorption. This principle is utilized in the combination of epinephrine with local anesthetics. Absorption of drugs implanted under the skin in a solid pellet form occurs slowly over a period of weeks or months; several hormones are effectively administered in this manner. (*See* review by Schou, 1961.)

Intramuscular. Drugs are often injected deep into muscle tissue. From this site, drugs in aqueous solution are rapidly absorbed by the diffusion processes described above. It is possible to dissolve or suspend drugs in oil and inject them intramuscu-

larly. This leads to a very slow and even rate of absorption. Drugs with low solubility, such as the repository penicillin preparations, are also slowly absorbed from intramuscular sites. Irritating substances that cannot be injected subcutaneously may be given by the intramuscular route.

Intraperitoneal. The peritoneal cavity offers a large absorbing surface from which drugs enter the circulation rapidly. Intraperitoneal injection is a common laboratory procedure, but it is seldom employed clinically. The dangers of infection and adhesions are too great to warrant the routine use of this route in man.

Intravenous. Drugs in aqueous solution can be introduced directly into the circulation, usually into a vein. The factors concerned in absorption are circumvented by intravenous injection, and the desired blood concentration of a drug is obtained with an accuracy and immediacy not possible by any other procedure. In some instances, as in the induction of surgical anesthesia by a barbiturate, the dose of a drug is not predetermined but is adjusted to the response of the patient. Also, certain irritating and hypertonic solutions can be given only in this manner, for the blood vessel walls are relatively insensitive and the drug, if injected slowly, is greatly diluted by the blood.

On the other hand, there are many dangers that attend intravenous injections. Unfavorable reactions are more prone to occur than when any other route is used. Once the drug is injected there is no retreat, whereas when subcutaneous administration is employed absorption can be stopped by occluding venous return from the area of injection. Repeated intravenous injections are dependent upon the patency of veins. Drugs in an oily vehicle or those that precipitate blood constituents or hemolyze erythrocytes should not be given by this route. Unless specifically indicated, drugs should never be given directly into the blood stream.

Intra-arterial. Occasionally a drug is injected directly into an artery in order to localize its effect in a particular tissue or organ. Antineoplastic agents are sometimes given in this manner for the treatment of localized tumors. This procedure requires great care, and its use should be reserved for experts.

Intrathecal. The blood-brain barrier and the blood–cerebrospinal fluid barrier often preclude or slow the entrance of drugs into the central nervous system (CNS). Therefore, when local and rapid effects of drugs on the meninges or cerebrospinal axis are desired, as in spinal anesthesia or acute CNS infections, drugs are sometimes injected directly into the spinal subarachnoid space.

Pulmonary Endothelium. Gaseous and volatile drugs may be inhaled; they are then absorbed through the pulmonary endothelium or mucous membranes of the respiratory tract and in this way gain rapid access to the circulation. The principles governing absorption and excretion of gases and vapors are discussed in Chapter 5. Also, solutions of drugs can be atomized and the fine droplets in air (aerosol) inhaled. Advantages are the almost instantaneous absorption of a drug into the blood and, in the case of pulmonary disease, local application of the drug at the desired site of action. For example, epinephrine can be given in this manner for the treatment of bronchial asthma. The main disadvantages are poor ability to regulate the dose, cumbersomeness of the methods of administration, and the fact that many gaseous and volatile drugs produce irritation of the pulmonary endothelium.

Mucous Membranes. Absorption of drugs takes place readily through many of the mucous membranes of the body other than those of the alimentary canal, and the more accessible ones are often employed for this purpose. Certain drugs can reach the circulation through the mucous membranes of the vagina, urethra, conjunctiva, nose, and oropharynx. In many instances, drugs are applied at these sites for their local action, but on occasion it is the systemic effect that is desired. In fact, local anesthetics may sometimes be absorbed so rapidly from the mucous membranes that they produce systemic toxicity.

Skin. Few drugs readily penetrate the intact skin. Absorption of those that do is proportional to their lipid solubility since the epidermis behaves as a lipoid barrier; the dermis, however, is freely permeable to many solutes. As a result, toxic effects can sometimes be produced by absorption through the skin of highly lipid-soluble substances (*e.g.*, a lipid-soluble insecticide in an organic solvent). Absorption through the skin can be enhanced by suspending the drug in an oily vehicle and rubbing the resulting preparation into the skin. This method of administration is known as *inunction.* Lipid-insoluble molecules and ions penetrate slowly. The rate of transfer of drugs that ionize, however, can be increased by a procedure known as *iontophore-*

sis. This is accomplished by passage of a galvanic current through a solution of drug applied to the skin underneath an electrode. The procedure is not often used, since large amounts of drugs are required, the dose given is difficult to determine, and the method is cumbersome.

DISTRIBUTION

After a drug is absorbed or injected into the blood stream, it can enter or pass through the various body fluid compartments —plasma, interstitial fluid, transcellular fluids, and cellular fluids. Some drugs cannot pass cell membranes and, therefore, are restricted in their distribution and in their sites of action, whereas others pass through cell membranes and thereby distribute throughout all fluid compartments. In addition, some drugs may accumulate in various areas as a result of binding, dissolving in fat, or active transport. The accumulation may be at the locus of action of the drug or, more often, in some other location. In the latter situation, the site of accumulation may serve as a storage depot for the drug.

Storage Depots. Many areas of the body serve as reservoirs for drugs because selective accumulation occurs at these sites. Stored drug is in equilibrium with that in plasma and is released as plasma concentrations are reduced. As a result, effective plasma levels of the drug are maintained for a longer period, and pharmacological effects are correspondingly prolonged. However, to obtain therapeutically effective levels initially, adequate priming doses must be given to saturate the binding sites. The following are the major drug storage sites in the body.

Plasma Proteins and Other Extracellular Depots. Upon entering the blood, a drug may be bound to plasma proteins. Ordinarily the binding is to albumin. However, other plasma proteins also bind drugs. The binding involves reversible bonds of the ionic, hydrogen, and van der Waals types. The extent of binding depends on the particular drug. For example, it may be high, as with phenylbutazone (98%); or low, as with barbital (5%); or practically nil, as with antipyrine. If affinity of a drug for the albumin is high, the plasma proteins may serve as a rather large storage depot for the drug. For example, suramin, an effective agent in the therapy of trypanosomiasis, is strongly bound to plasma protein, and a single dose of the drug confers protection against infection for 3 months or more.

Some drugs are stored in connective tissue because they are bound to the strongly ionic groups of the mucopolysaccharides. Bone may also be a reservoir for drugs, and substances such as heavy metals and tetracyclines are stored there. The nature of the accumulation in bone is not known, but it probably is due to adsorption of the substances to the bone-crystal surface or incorporation into the crystal lattice.

Cellular Depots of Drugs. Many drugs accumulate in higher concentrations in cells than in the extracellular fluids. If the intracellular concentration is high, the tissue involved may serve as a large storage depot. Accumulation in cells may be brought about by active transport or by binding to tissue constituents. Tissue binding of drugs usually occurs with proteins, phospholipids, or nucleoproteins and is generally reversible. A classical example of a drug that binds to tissue constituents is the antimalarial agent quinacrine. This drug may achieve a concentration in liver 2000 times that of the plasma level 4 hours after a single dose. After chronic administration, the accumulation is even more pronounced and may amount to 22,000 times the plasma level. The binding occurs in the nuclei, probably with nucleoproteins.

Fat as a Depot for Drugs. Many drugs have a high lipid solubility and are stored by physical solution in the neutral fat. In obese persons, the fat content of the body may be as high as 50%, and even in starvation it makes up 10% of body weight; hence, fat can serve as an important depot for storage of lipid-soluble drugs. The majority of a drug with a high lipid:water partition coefficient may enter fat. For example, as much as 70% of the highly lipid-soluble barbiturate thiopental may be present in body fat 3 hours after administration.

Transcellular Depots of Drugs. Drugs also cross epithelial cells into the transcellular fluids and may accumulate in these fluids. The major transcellular depot in the body is the gastrointestinal tract. A drug that is poorly soluble in gastrointestinal fluid will be slowly absorbed and serve as a

reservoir for maintaining the level in the plasma and increasing its duration in the body. Weak bases are passively concentrated in the stomach from the blood, because of the large pH differential between the two fluids; the gut thus serves as a storage depot for such compounds.

Drugs do not generally accumulate in the *cerebrospinal fluid,* because no protein is present for binding, they exit relatively rapidly by way of the arachnoid villi, some are actively transported out of cerebrospinal fluid, and there is only a small pH gradient across the choroid plexus. Other transcellular fluids, such as *aqueous humor, endolymph,* and *joint fluids,* do not generally accumulate drugs and constitute only minor storage depots in the body. The *luminal fluid* of the *thyroid* serves as the major storage depot for iodine in the body and can concentrate drugs such as the perchlorate and some other monovalent anions.

Passage of Drugs into and across Cells. The passage of drugs across cell membranes involves the same factors discussed above for membranes in general. Weak electrolytes penetrate cells by simple diffusion in the nonionized form in proportion to their lipid: water partition coefficient and are distributed between extracellular and intracellular fluids in proportion to the pH difference of the two fluids. However, since the pH difference between intracellular and extracellular fluids is small (7.0 versus 7.4), the concentration gradient across the membrane is also small. Weak bases are concentrated slightly inside of cells, while the concentration of weak acids is slightly lower in the cells than in extracellular fluids. Lowering the pH of extracellular fluid increases the intracellular concentration of weak acids and decreases that of weak bases, provided that the pH change does not simultaneously affect the binding, biotransformation, or excretion of the drug. Elevating the pH produces the opposite effects. Nonelectrolytes enter cells by diffusion and generally in proportion to their lipid solubility, but small molecules such as urea penetrate through aqueous channels in the membrane. Penetration of strong acids and bases that are completely ionized depends upon the permeability of, and the potential difference across, the cell membrane.

The penetration of drugs into subcellular particles such as mitochondria, although little studied, appears to be an important aspect of drug distribution and action. The mitochondrial membrane is lipoid in nature, and penetration of drugs follows the same principles as for cell membranes.

Penetration into Central Nervous System and Cerebrospinal Fluid. The entrance of drugs into the CNS and the cerebrospinal fluid is a special aspect of cellular penetration; however, in general, it follows the same principles as for transfer across other cells. The blood-brain barrier is located not at the surface of brain cells but between the plasma and extracellular space of the brain, at the basement membrane of capillary endothelial cells. Transfer of drugs across neuronal cell membranes is like that across any other cell membrane. The blood–cerebrospinal fluid barrier is located at the choroid plexus. Lipid-insoluble drugs and inorganic and organic ions enter the brain much more slowly than do lipid-soluble substances. Their rate of entrance is proportional to the size of the molecule; large molecules like inulin penetrate slowly, whereas small ions like chloride or small nonelectrolytes like urea penetrate more rapidly, but still slowly as compared to the rate in other tissues. The choroid plexus is also slowly permeable to small ions and lipid-insoluble substances but poorly permeable or impermeable to large lipid-insoluble substances.

The routes of exit of drugs from the cerebrospinal fluid differ from the routes of entrance. All drugs and many endogenous metabolites, regardless of lipid solubility or molecular size (including molecules as large as plasma albumin), leave the cerebrospinal fluid by flow through the arachnoid villi. The speed of exit is the same for all the substances and depends on the rate of the bulk flow of cerebrospinal fluid. In addition, if the drug is lipid soluble, it can exit by the same route it entered, namely, by diffusion across the lipid portions of the blood–cerebrospinal fluid boundary. Drugs can also diffuse into and out of the brain through the blood-brain boundary; exit by this route is aided by bulk flow of cerebrospinal fluid through the brain and into the capillaries. In addition, certain drugs and endogenous metabolites may be removed from the cerebrospinal fluid by specialized, active transport processes, similar to those that exist for organic ions in the renal tubules (*see* below). (For summaries of drug penetration into and out of the CNS, *see* Rall and Zubrod, 1962; Schanker, 1962.)

Placental Transfer of Drugs. A knowledge of the principles of transfer of drugs across the placenta is important since drugs can exert toxic effects on the fetus and may induce congenital anomalies. The transfer occurs primarily by simple diffusion; carrier-mediated transport is generally restricted to endogenous substrates. Nonionized drugs of high fat solubility readily enter the fetal blood from the maternal circulation. Penetration is least with drugs possessing a high

degree of dissociation and/or low lipid solubility. (For summaries of placental transfer of drugs, *see* Schanker, 1962; Symposium, 1964; Goldstein *et al.*, 1968.)

Redistribution. Although termination of drug effect is usually accomplished by biotransformation and excretion, it may also result from redistribution of the drug from its site of action into other tissues; however, it is stored there in an active form and its ultimate elimination from the body is still dependent on biotransformation and excretion. If the inital dose saturates the storage site, a subsequent dose of the drug may produce a prolonged effect. The factors involved in redistribution of drugs have been extensively studied for thiopental and are described in Chapter 9.

BIOTRANSFORMATION

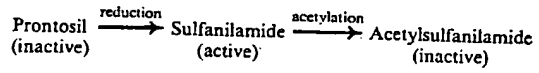
Many drugs are lipid-soluble, weak organic acids and bases that are readily reabsorbed in the renal tubules. To be excreted more rapidly, they must be transformed into more polar compounds. These more ionized, less lipid-soluble metabolites are also less able to bind to plasma and tissue proteins, less likely to be stored in fat depots, and less able to penetrate cell membranes. Thus, this type of biotransformation usually results in inactivation of the drug. Occasionally, however, activation may result, or an active drug may be transformed into a metabolite that is also active. In such cases, termination of action takes place by further biotransformation or by excretion of the active metabolite in the urine.

General Considerations. Although much of the information concerning the biotransformation of drugs is based upon observations in laboratory animals, many clinical studies demonstrate that similar mechanisms occur in man. However, the rates at which the reactions proceed in the various species are often very different; a drug may be rapidly inactivated and have a short duration of action in animals, yet be much more slowly inactivated and have a long duration of action in man.

The chemical reactions concerned in the biotransformation of drugs can be classified as *nonsynthetic* and *synthetic*. The nonsynthetic reactions involve oxidation, reduction, or hydrolysis, and may result in activation, change in activity, or inactivation of the parent drug. The synthetic reactions, also called conjugation, involve coupling between the drug or its metabolite and an endogenous substrate that is usually a carbohydrate, an amino acid, derivatives of these or an inorganic sulfate. Synthetic reactions almost invariably result in inactivation of the parent drug.

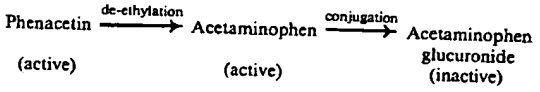
Various patterns of biotransformations involving nonsynthetic and synthetic reactions may be distinguished. (For summaries of drug biotransformations, *see* Symposium, 1962; Williams, 1959, 1963; Goldstein *et al.*, 1968; many others.)

One type may be exemplified by the following reactions:



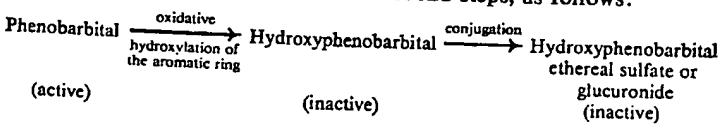
In this case, the inactive parent drug is converted to an active chemotherapeutic agent by a reductive process, and the active drug is subsequently inactivated by a synthetic reaction involving acetylation. The sojourn of the active drug in the body depends upon the relative rates of activation and inactivation.

A second pattern of biotransformation is depicted in the following scheme:



This biotransformation involves change of the parent drug to a metabolite that is also active until conjugated. The first step may occur without the synthetic second step. For example, the anticonvulsant trimethadione is converted to the active metabolite 5,5-dimethyl-2,4-oxazolidinedione (DMO) by oxidative N-demethylation. DMO is not further metabolized but is slowly excreted in the urine. A change in the type of biological activity may also occur. For example, aniline, a highly toxic substance with pharmacological effects similar to those of acetanilid and phenacetin, is converted in the body to a metabolite that causes methemoglobin formation.

Inactivation may also occur in both the first and second steps, as follows:



The first phase of inactivation may be oxidative, reductive, or hydrolytic. Reactions of this type are extremely common and are often responsible for the termination of drug activity. The synthetic step may not be present in some cases. For example, procaine is inactivated by hydrolytic cleavage to *p*-aminobenzoic acid and diethylaminoethanol.

Another biotransformation, usually resulting in inactivation, involves only the synthetic reaction. A typical example is the following:

$$\underset{\text{(active)}}{\text{Salicylamide}} \xrightarrow{\text{conjugation}} \underset{\text{(inactive)}}{\text{Salicylamide glucuronide}}$$

These reactions are often called detoxification, and many drugs are inactivated in this manner. Rarely, a change in activity may occur.

Specific Processes. Biotransformation of drugs occurs mainly in the liver, but also takes place in plasma, kidney, and other tissues.

Oxidation. An important group of oxidative enzymes (called mixed-function oxidases) is located in hepatic microsomes. The reactions catalyzed by these oxidative enzymes include N- and O-dealkylation, aromatic ring and side chain hydroxylation, sulfoxide formation, N-oxidation, N-hydroxylation, and deamination of primary and secondary amines. The replacement of a sulfur by an oxygen atom (desulfuration) can also occur. Thus, parathion, which contains a —P=S group and is inactive, is converted to paraoxon, which contains a —P=O group and is an insecticide with anticholinesterase properties. NADPH and molecular oxygen are essential in most of these reactions, as discussed below. Some drug oxidations are mediated by enzymes other than those of the hepatic microsomal system—for example, oxidation of alcohols in the soluble fraction of the liver by *dehydrogenation*. In addition, monoamine and diamine oxidases are mitochondrial enzymes, found especially in liver, kidney, intestine, and nervous tissue, that oxidatively deaminate several naturally occurring amines as well as a number of drugs. Also, many halogenated compounds used as insecticides and industrial solvents are *dehalogenated* in the soluble fraction of the liver. The reactions include replacement of a halide atom by a hydroxyl group, loss of hydrogen halide, and replacement of halide by an acetylcysteine group (mercapturic acid formation).

Reduction. Hepatic microsomes and some other tissues also contain enzymes that catalyze the reduction of nitro groups and the cleavage and reduction of the azo linkage. The reactions are catalyzed by flavoproteins that require NADPH as the hydrogen donor and in some cases cytochrome P-450, as discussed below. Examples are the nitro reduction of chloramphenicol and the azo reduction of PRONTOSIL. The conversion of chloral hydrate to trichloroethanol is a reduction reaction catalyzed by alcohol dehydrogenase.

Hydrolysis. These reactions involve deamidation by hepatic enzymes and de-esterification by esterases located in plasma, soluble fraction of liver, and many other tissues. Examples include the hydrolysis of procaine, procainamide, meperidine, acetylcholine, and many other esters and amides. Peptidases in plasma, erythrocytes, and many other tissues are involved in the biotransformations of the biologically active polypeptides.

Synthesis (Conjugation). A variety of synthetic reactions are involved in the inactivation of drugs. The substances are conjugated to form inactive, highly ionized, water-soluble substances that are readily excreted in the urine. These reactions are termed synthetic, since they are endothermic and require a source of energy, usually adenosinetriphosphate (ATP). The reaction involves activation of either the drug or the conjugating agent. Conjugating enzymes occur mainly in the liver but also in other tissues, particularly the kidney. The major conjugation reactions are: *glucuronide* formation, an important pathway in the metabolism of phenols, alcohols, and carboxylic acids, a process that involves the formation of uridine diphosphate–glucuronic acid (UDPGA), which serves as a donor of glucuronic acid to the drug acceptors and is catalyzed by various transferase enzymes; *ribonucleoside* and *ribonucleotide* synthesis, usually with analogs of purines and pyrimidines to form biologically active anticancer agents, reactions catalyzed by the same enzymes (soluble fraction) responsible for formation of the naturally occurring nucleosides and nucleotides; *sulfate* conjugation, mainly with phenols and involving formation of an active sulfate donor by reaction of inorganic sulfate with ATP; *acetylation*, usually involving the conjugation of acetyl coenzyme A (CoA) with an acceptor amine, such as sulfanilamide; *O-*, *S-*, and *N-methylation*, with certain types of phenolic compounds, nicotinic acid, and epinephrine and norepinephrine, the methyl group being supplied by S-methyladenosylmethionine; and *glycine* conjugation, with aromatic acids such as benzoic and salicylic acids and involving the formation of a CoA-drug intermediate.

Sites and Mechanisms of Biotransformation. Drugs are metabolized by a variety of enzymes involved in intermediary metabolism. For example, some alcohols are inactivated by alcohol dehydrogenase; succinylcholine, by plasma pseudocholinesterase; 6-mercaptopurine, by xanthine oxidase. Administered biogenic amines and their precursors are biotransformed by the enzymes (monoamine oxidase, tyrosine hydroxylase, etc.) involved in the normal synthesis and metabolism of these compounds. However, the majority of drugs are metabolized by the hepatic microsomal enzymes.

Microsomal Drug-Metabolizing System. The enzyme systems concerned in the biotransformation of many drugs and also of steroid hormones and lipids appear to be located in the hepatic endoplasmic reticulum (ergastoplasm). Fragments of this network are isolated from liver homogenates by various technics in the fraction generally called *microsomes*. The microsomal enzymes catalyze many of the nonsynthetic and synthetic biotransformations described above. Since so many drugs are metabolized by this system, it is important to consider these enzymes in some detail and to note the factors that influence their activity.

The endoplasmic reticulum resembles a kind of pipeline or canal system in the cell and may function in the transport of substances from one area of a cell to another; it is continuous with the cell membrane and with the nuclear membrane. As identified by electron microscopy, the reticulum consists mainly of a membrane that often bears small ribonucleoprotein particles, called *Palade granules*, which cause the reticulum to have a rough surface. Only the smooth-surfaced microsomes are thought to possess enzymes that can metabolize drugs. The rough-surfaced microsomes are concerned with protein synthesis.

Most of the oxidative and some of the reductive drug-metabolizing enzymes in the hepatic microsomes are unusual in that they require NADPH and molecular oxygen for their action. Electron transfer appears to involve three carriers, as depicted in Figure 1–2.

NADPH is oxidized by a flavoprotein enzyme, NADPH–cytochrome C reductase, which in turn appears to be reoxidized by an incompletely characterized enzyme thought to be a non-heme iron (NHI) protein. This reduced carrier then is reoxidized by cytochrome P-450, so named because it absorbs light at 450 mμ when exposed to carbon monoxide, a property that makes possible its analytical determination. Furthermore, carbon monoxide also blocks the metabolism of many drugs by the hepatic microsomal system. The drug to be metabolized appears to be bound firmly to oxidized P-450. This drug-oxidized P-450 complex, after reduction by the NHI protein, reacts directly with molecular oxygen to form a drug-oxidized P-450-O complex and water. A hydroxyl group is transferred to the drug, and oxidized P-450 is regenerated as the oxidized drug is released.

The rate of drug biotransformation appears to be related not only to the amount of drug–P-450 complex but also to its rate of enzymatic reduction. The amount of cytochrome P-450 in liver varies from species to species, strain to strain, and even individual to individual; this might explain the large variability in rate of metabolism of a particular drug observed in man. When the rate of a drug oxidation is increased by prior treatment with an inducing agent such as phenobarbital (*see* below), the amount of cytochrome P-450 also increases and this can explain the enhancing effect of phenobarbital on drug-oxidation reactions. There is ample spectral evidence to support the concept that drugs bind directly to cytochrome P-450. Apparently, this carrier is necessary, not only for active oxidation of a drug but also for the binding of the microsomes of the compound to be oxidized.

The reduction of some nitro and azo groups by the liver microsomes is also catalyzed by this system; in these reactions, the drug is first oxidized to an intermediate, which is then reduced by the H ions from NADPH. These hepatic enzymes also function for the inactivation of endogenous steroid hormones and the oxidation of fatty acids. As mentioned below, many drugs compete with these steroids, and possibly the fatty acids, for the oxidative enzymes involved. (*See* Symposium, 1965; Gillette, 1966; Conney, 1967; Goldstein *et al.*, 1968; many others.)

Lipid solubility is an important, but not the only, requirement for a drug to be metabolized by the hepatic microsomes since this property favors the penetration of a drug into the endoplasmic reticulum and the binding with cytochrome P-450. Endogenous substrates except steroids and fatty acids are not affected since most are polar, water-soluble compounds. The evolutionary development of the drug-metabolizing system in hepatic microsomes is a fascinating story in comparative pharmacology that is beyond the scope of this chapter. Summaries of this aspect have been presented by Brodie and Maickel in a symposium (1962).

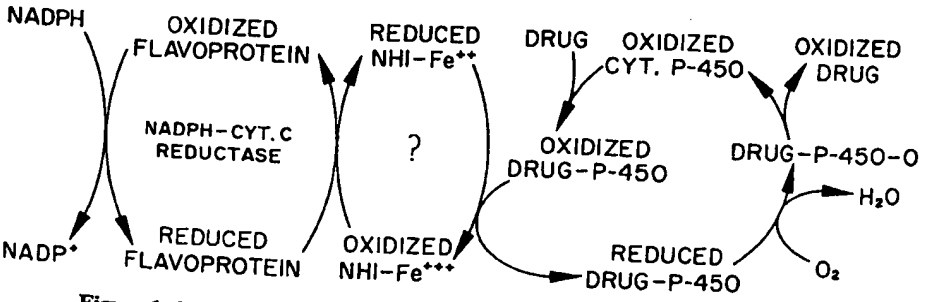


Figure 1–2. *Electron transfer in the microsomal drug-metabolizing system.*

The activity of the drug-metabolizing enzymes in hepatic microsomes, as well as the structure and amount of smooth-surfaced endoplasmic reticulum, is influenced markedly by the administration of various drugs and hormones and by the age, sex, strain, temperature, nutritional status, and pathological state of the animal. Some of these factors are discussed below.

Inhibitors. The effects of many drugs are enhanced and prolonged by interference with their enzymatic destruction by hepatic microsomal enzymes. The first such compound described was SKF 525A (β-diethylaminoethyl-2,2-diphenylpentanoate), a compound with no other appreciable pharmacological effects. Many drugs structurally related to SKF 525A and various other compounds produce similar inhibition of microsomal enzymes.

In man, a number of drugs inhibit the metabolism of other drugs. For example, phenylbutazone and bishydroxycoumarin inhibit the metabolic inactivation of tolbutamide and profound hypoglycemia occurs if one of these drugs is administered concurrently with tolbutamide. Similarly, bishydroxycoumarin and disulfiram inhibit the metabolism of diphenylhydantoin and can cause accumulation of the anticonvulsant to toxic levels. In addition, some agents are known to enhance the anticoagulant effect of bishydroxycoumarin by inhibition of its metabolism; serious hemorrhage can result if care is not taken when these drugs are given concurrently. These inhibitors appear to act in a variety of ways: interference with NADPH-generating systems, inhibition of reduction of P-450, and blockade of the transfer of active oxygen from P-450 to the drug substrate. They also may inhibit by more than one mechanism. For example, SKF 525A is converted to a substance that inactivates P-450, but it also presumably combines with the active site of an N-dealkylase in hepatic microsomes since the inhibition is competitive; it is also a competitive antagonist of microsomal and plasma esterases. (*See* Gillette, 1966.)

Stimulators. Activity of the hepatic microsomal enzymes may also be increased by the administration of drugs. Prior treatment of experimental animals with various agents increases their ability to metabolize both the administered drug as well as other related and unrelated compounds and some endogenous substrates. After prior treatment of rats with phenobarbital, the metabolism of zoxazolamine, hexobarbital, and phenobarbital itself is increased and their pharmacological effects are proportionately reduced. Various carcinogenic hydrocarbons, steroid hormones, and at least 200 other agents exert similar effects, but still others drugs have no such enhancing effect on enzyme activity. The increased activity of the microsomal enzymes is probably due to enhanced synthesis of cytochrome P-450, NADPH–cytochrome C reductase, and other enzymes involved in the metabolism of drugs. This probability is supported by the facts that ethionine, puromycin, and actinomycin, which interfere with protein synthesis, block the effect and that the enhancement of microsomal enzyme activity does not occur when the stimulating agents are added to the enzymes *in vitro*. Also, inducers increase RNA polymerase activity. Enzyme induction by some drugs occurs to a limited extent in hepatic mitochondria and in kidney, gastrointestinal tract, adrenal, lung, and, occasionally, other tissues as well.

The observations that one drug can stimulate its own metabolism, that of another drug, or of normal body constituents have wide implications for chronic toxicity tests, crossover drug studies in animals and man, chronic drug therapy with single or multiple drugs, and the development of tolerance to drugs. In each situation, the pharmacological effects of the second dose or of the second drug administered will be modified by the first, since its metabolism will be altered by stimulation of the drug-metabolizing enzymes.

Drug-induced stimulation of microsomal enzyme activity occurs in man and plays an important role in drug therapy. Enzyme induction is responsible for some altered pharmacological effects that occur during chronic medication. Many examples have been described, but only a few will be presented here to illustrate this important concept. Other examples are discussed throughout the text. (For excellent reviews, also *see* Burns, 1964; Burns and Conney, 1965; Conney, 1967, 1969.) Concurrent administration of phenobarbital and bishydroxycoumarin results in lower plasma levels of bishydroxycoumarin and less anticoagulant effect than when bishydroxycoumarin is administered alone. Even larger doses of the anticoagulant cannot maintain adequate blood levels of the drug in the presence of phenobarbital. If phenobarbital is discontinued, the plasma level and anticoagulant effect of bishydroxycoumarin increase to such an extent that severe bleeding may result. Thus, when

concurrent therapy with a stimulator of drug metabolism and another drug is undertaken, extreme care must be observed when the dose of either is increased or reduced. Phenobarbital, in addition, stimulates the metabolism and lowers the blood levels of diphenylhydantoin, griseofulvin, and aminopyrine. As a result of enzyme induction by drugs, the hydroxylation of androgens, estrogens, progestational steroids, and glucocorticoids, and the conjugation of bilirubin are increased. Chronic administration of a drug may also stimulate its own metabolism and thereby cause tolerance. Environmental carcinogens, insecticides, and cigarette smoking have been shown to induce hepatic drug-metabolizing enzymes in experimental animals. The implications of this effect for man are now being assessed. Cigarette smoking, for example, enhances the metabolism of nicotine in man, and this may explain the tolerance to nicotine that occurs in smokers. Whether smoking also stimulates the metabolism of commonly used drugs in man is an important, but unanswered, question. The inductive effect of phenobarbital on glucuronide conjugation has been used to advantage in the treatment of hyperbilirubinemia in infants with congenital nonhemolytic jaundice; phenobarbital treatment lowers the free plasma bilirubin concentration, and the jaundice disappears. In such infants there is a genetic deficiency in formation of glucuronides, and phenobarbital treatment enhances the activity of glucuronyl transferase that carries out this reaction.

EXCRETION

The kidney is the most important organ for drug excretion. Drugs and their metabolites excreted in the feces are derived either from unabsorbed orally ingested drug or from metabolites excreted in the bile and not reabsorbed from the intestinal tract. Excretion of drugs in milk is important not because of the amounts eliminated but because the excreted drugs are potential sources of unwanted pharmacological effects or toxicity in the nursing infant. Pulmonary excretion is of importance mainly for the elimination of anesthetic gases and vapors, but some metabolites of toxic substances are excreted by this route. Factors affecting pulmonary excretion are discussed in Chapter 5.

Drugs are eliminated from the body either unchanged or as metabolites. Generally, the more polar compounds are excreted unchanged. The less polar, lipid-soluble drugs, however, are not readily eliminated until they are metabolized to more polar, less lipid-soluble compounds.

Renal Excretion. Excretion of drugs in the urine involves three processes: passive glomerular filtration, active tubular secretion and reabsorption, and passive tubular diffusion. The amount of drug entering the tubular lumen by *filtration* is dependent on the filtration rate and the degree of plasma protein binding. In the proximal renal tubule, strong organic acids and bases are added to the glomerular filtrate by active, carrier-mediated tubular *secretion*. Many organic acids, such as penicillin, are transported by the system that secretes naturally occurring substances such as uric acid; many organic bases, such as tetraethylammonium, are transported by another system that secretes choline, histamine, and other naturally occurring bases. Ionic forms of some weak electrolytes, such as salicylate, chlorothiazide, and quinine, are also secreted into the tubular lumen by these mechanisms. The characteristics of tubular transport systems are described in detail in Chapter 41.

In the proximal and distal tubules, the nonionized forms of weak acids and bases undergo reabsorption or excretion by *passive diffusion*. By definition, a passive diffusion mechanism is potentially bidirectional, and drugs may diffuse across tubular cells in either direction, depending upon the concentrations of the drug and the pH on the two sides of the tubular cells. Under normal conditions, even when the pH gradient in the distal tubule favors diffusion into the urine, the net effect is reabsorption, since the bulk of the drug will diffuse out of the urine as reabsorption of strong electrolyte and water creates a concentration gradient of the nonionized form in the direction of urine to blood. Passive reabsorption of the ionized form may also occur, but the proportion is considerably less than that of the nonionized form. When the tubular urine is more alkaline than plasma, weak acids are excreted more rapidly, primarily because of a decrease in net passive reabsorption. When the tubular urine is more acid than plasma, the excretion of weak acids is reduced. The effects of alkalinization and acidification of the urine on the excretion of weak bases are the opposite of those on the excretion of weak acids. In poisoning, the excretion of some drugs can be hastened by appropriate alkalinization or acidification of the urine. (*See*

Orloff and Berliner, 1961; Mudge and Weiner, 1963.)

Hepatic and Fecal Excretion. Many metabolites of drugs formed in the liver are excreted into the intestinal tract in the *bile*. The metabolite may be excreted in the feces, but the greater portion is usually reabsorbed into the blood and ultimately excreted in the urine. For example, diphenylhydantoin is metabolized in the liver, chiefly to hydroxydiphenylhydantoin; all of this metabolite enters the bile and hence into the intestinal tract, but it is subsequently reabsorbed and is ultimately excreted in the urine. The metabolite of colchicine, in contrast, is secreted in the bile and excreted only in feces, since absorption across the intestinal mucosa does not occur. Both strong organic acids and strong organic bases are actively transported from hepatic cells into bile by processes similar to those that transport these same substances across renal tubules.

Excretion by Other Routes. Excretion of drugs into *sweat* and *saliva* takes place by similar mechanisms, but both routes are quantitatively unimportant. Excretion is dependent mainly upon diffusion of the nonionized, lipid-soluble form of drugs through the epithelial cells of the glands, since the ionized forms pass only slowly into these secretions. Thus, pK_a of the drug and pH of the primary secretion formed in the acini of the glands are important determinants of secretion rate. Nonionic reabsorption of the drug from the primary secretion probably also occurs in the ducts of the glands, and this process is dependent on the pH of the fluid in the duct lumen. It is not known whether active secretion of drug occurs across the ducts of the gland. Lipid-insoluble compounds, such as urea and glycerol, appear to enter saliva and sweat at rates proportional to their molecular weight, presumably by filtration through aqueous channels in the membrane. Drugs excreted in the saliva enter the mouth, where they are usually swallowed. Their fate thereafter is the same as that of drugs taken orally.

The same principles apply to excretion of drugs in *milk*. Since milk is more acidic than plasma, basic compounds may be concentrated in this fluid. In contrast, the concentration of acidic compounds in milk is lower than in plasma. Nonelectrolytes, such as ethanol, urea, and antipyrine, readily enter milk and reach the same concentration as in plasma, independently of the pH of the milk. (*See* summary by Stowe and Plaa, 1968.)

MECHANISMS OF DRUG ACTION

The most fundamental aspect of pharmacodynamics is that which deals with the mechanisms of drug action. Before summarizing some general concepts pertaining to this fascinating subject, it is helpful to emphasize the distinction between drug action and drug effect, and clearly to define the objectives of analysis of drug action.

Although often considered as synonyms, the terms *action* and *effect* have useful pharmacological connotations that should be preserved. With certain obvious exceptions, most drugs are thought to produce their effects by combining with enzymes, cell membranes, or other specialized functional components of cells. Drug-cell interaction is presumed to alter the function of the cell component and thereby initiate the series of biochemical and physiological changes that are characteristic of the drug. Only the initial consequence of drug-cell combination is correctly termed the *action* of the drug; the remaining events are properly called drug *effects*. The objectives of analysis of drug action are identification of the primary action, delineation of the details of the chemical reaction between drug and cell; and characterization of the full action-effects sequence. Only the complete analysis provides a truly satisfactory basis for the therapeutic use of the drug, but this is an ideal that is infrequently attained.

Structure-Activity Relationship. The actions of a drug are intimately related to its chemical structure. The relationship is frequently quite specific, and relatively minor modifications in the drug molecule may result in major changes in pharmacological properties. Exploitation of structure-activity relationship has led to the synthesis of many valuable therapeutic agents. Since changes in molecular configuration need not alter all actions and effects of a drug equally, it is sometimes possible to develop a congener with a more favorable therapeutic index or more acceptable secondary characteristics than those of the parent drug. In addition, effective therapeutic agents have been fashioned by developing structurally related competitive antagonists of other drugs or of endogenous substances known to be important in biochemical or physiological function.

Sometimes the structure-activity relation-

ship appears quite broad. For example, many chemically dissimilar drugs exhibit local anesthetic activity, and compounds of totally different chemical structure produce similar CNS depressant effects. These examples do not negate the significance of the structure-activity relationship, but merely emphasize that much remains to be learned of the basic mechanisms of action of most drugs and that they may produce overtly similar effects by more than a single mechanism.

Drug Receptors. The cell component directly involved in the initial action of a drug is usually termed its *receptive substance* or, simply, its *receptor*. The chemical groups that participate in drug-receptor combination and the adjacent portions of the receptor that favor or hinder access of the drug to these active groups are known as *receptor groups* or *receptor sites*. The formation of the drug-receptor complex, the initial action of the drug, and the early events in the action-effects sequence are sometimes referred to collectively as the *receptor process*. Drug-cell interactions that do not initiate drug action, such as the binding of drugs to plasma and cell proteins and to enzymes concerned with biotransformation and transport of drugs, are said to involve *secondary receptors, silent receptors, sites of loss, storage sites,* or *drug acceptors.*

Receptors and Theories of Drug Action. As early as 1878, even before he coined the term *receptive substance,* Langley suggested that drug-cell combinations, and hence the actions and effects of drugs, were probably governed by the law of mass action. This view was extensively developed by A. J. Clark in the 1920s, and it remains the keystone of most theories of drug action. Thus, theories of drug action are quite similar to theories of enzyme action and are essentially identical when a drug serves as an antimetabolite or enzyme inhibitor.

A drug that combines with receptors and initiates an action-effects sequence is said to possess both *affinity* and *efficacy* (or *intrinsic activity*) and is termed an *agonist;* one that combines with the same receptors but fails to initiate drug action is considered to lack efficacy and acts as a *competitive antagonist.* An agonist that produces a smaller maximal effect than do other agonists that act on the same receptor is said to have intermediate efficacy and is termed a *partial agonist.* The effects of a partial agonist and those of a full agonist acting on the same receptor may be additive, or the partial agonist may antagonize the full agonist, depending upon their relative concentrations. Many examples of partial agonists have been noted among the autonomic agents, and nalorphine and related so-called opioid antagonists appear to be partial agonists for certain opioid receptors. Why various drug-receptor combinations differ in their ability to initiate drug action remains to be explained.

The major value of receptor theory is that of providing a conceptual framework for analysis of mechanism of drug action. However, it must be emphasized that drug action is defined not by an equation derived from receptor theory that relates dose and effect or that describes the pattern of drug interaction, but only by identification of the role of drug receptors in normal cellular function and by characterization of the action effects sequence.

For certain applications of receptor theory, it is necessary to assume some relationship between drug-receptor interaction and intensity of drug effect. In the classical receptor theory developed by Clark, it was assumed that drug effect is proportional to the fraction of receptors occupied by drug and that maximal effect results when all receptors are occupied. Neither of these assumptions is likely, and subsequent modifications of *occupation theory* have assumed other relationships between receptor occupation and drug effect and have permitted the possibility that maximal effect may be achieved when only a portion of receptors is occupied. The latter concept is described as that of *spare receptors* or *receptor reserve.* It has also been proposed that drug effect may be a function not of receptor occupation but of the rate of drug-receptor combination. This *rate theory* is attractive because it relates drug efficacy to the rate of dissociation of the drug-receptor complex and explains certain other aspects of the time course of drug effect.

Although receptors for most drugs have yet to be identified, there is little doubt that drug-cell combinations that obey mass-law kinetics are involved in drug actions. The many discrete relationships between chemical structure and biological activity and the competitive interaction of chemically similar drugs are difficult to explain except in these terms. Receptor groups, like the active centers of enzymes, are thought to be carboxyl, amino, sulfhydryl, phosphate, and similar reactive groups

spatially oriented in a pattern complementary to that of the drugs with which they react. The binding of drug to receptor is thought to be accomplished mainly by ionic and other relatively weak, reversible bonds. Occasionally, firm covalent bonds are involved, and the drug effect is only very slowly reversible.

Classification of Receptors and Drug Effects. The statement that a drug activates or blocks specified drug receptors should not be mistaken as a description of its mechanism of action. Drug receptors are classified on the basis of the effect, or lack of effect, of selective antagonists and by the relative potencies of representative agonists. For example, the effects of acetylcholine that mimic those of the alkaloid muscarine and that are selectively antagonized by atropine are termed *muscarinic effects.* Other effects of acetylcholine that mimic those of nicotine and that are not readily antagonized by atropine but are selectively blocked by other drugs are described as *nicotinic effects.* By *extension,* these two types of cholinergic effects are said to be mediated by muscarinic or nicotinic receptors. Such classification of receptors contributes little to delineation of mechanism of drug action. Nevertheless, since the effects and receptors in the various tissues have been classified, a statement that a drug activates or blocks a specified type of receptor is a succinct summary not only of its spectrum of effects but also of the drugs that it will antagonize, or that will antagonize it. The use of receptor terminology to describe drug effects has been developed most extensively in connection with neurotransmitters and autonomic drugs, but the same concepts apply to other classes of drugs. However, receptor terminology is useful only if the drugs chosen for classifying drug receptors are selected in a meaningful manner, and if the effects of these drugs have been well characterized.

Sites of Action. Some drugs act only on certain cells, tissues, or organs and thereby exert *localized* effects. Other drugs act on most cells of the body and thus exert *generalized* effects. Localization of drug effect does not necessarily depend upon selective distribution of the drug. Some drugs act *extracellularly;* others, at the *cell surface;* and still others, *intracellularly.*

Certain drugs act *directly* on effector cells; others influence effector cells *indirectly.* A substance may lower blood pressure directly by inhibition of vascular smooth muscle, or indirectly by acting on the vasomotor center or on autonomic ganglia. Drugs may also produce their effects indirectly by promoting or preventing the action of another substance. Many of the effects of neostigmine are due to the preservation of acetylcholine at neuroeffector junctions, as a result of inhibition of the enzyme that normally destroys this neurotransmitter; and many of the effects of atropine are the result of competition with acetylcholine for its effector sites.

Biochemical and Biophysical Mechanisms of Action. The clinical effects of a drug can usually be described in terms of alterations of physiological function, and they can often be correlated with biochemical and biophysical effects of the agent. However, the primary actions of drugs have been elucidated in relatively few cases. Not infrequently, analysis of drug action is limited by available physiological and biochemical knowledge. Elucidation of basic cellular function and further exploration of drug action then proceed in parallel, often with the drug serving as an indispensable tool.

There are a few instances in which the mechanism of drug action is evident. Magnesium sulfate acts as a cathartic because the magnesium and sulfate ions are poorly absorbed and, therefore, exert an osmotic force that retains water in the lumen of the bowel. Also, dimercaprol (BAL) and other agents are useful in therapy of heavy-metal intoxication because they chelate with these metals to render them nontoxic.

Drugs may act by influencing the bound forms of endogenous, physiologically active substances. For example, the pharmacological effects of some drugs have been traced to the displacement of hormones, such as thyroxine, estradiol, and cortisol, from their binding sites on plasma proteins. Similarly, the pharmacological effects of tyramine result from *release* of norepinephrine from granules in adrenergic nerve terminals, and some antiadrenergic agents prevent the release of stored mediator. Other antiadrenergic drugs inhibit binding or decrease synthesis of norepinephrine and *deplete* the neurotransmitter from the nerve endings.

Drugs may also act by influencing any of the steps involved in maintenance of normal cell function. Thus, they may enhance or prevent the entrance into the cell of substances necessary for

energy production, synthetic reactions, or maintenance of the osmotic and electrical properties of the cell. These changes may be brought about either by interactions with specific drug receptors or by changing the structure of the surrounding solvent without interacting with the cells directly; general anesthetics may act in this latter way. *Transport* of glucose into cells is enhanced by insulin, the uptake of choline by nerve cells is blocked by hemicholinium, active transport of sodium and potassium is inhibited by digitalis, and the *permeability* of the neuromuscular junction to ions is increased by acetylcholine. Many drugs inhibit or enhance *enzyme* activity or influence energy production. For example, epinephrine enhances liver phosphorylase activity by accelerating the formation of cyclic adenosine-3',5'-monophosphate through the activation of the enzyme adenyl cyclase. Agents such as salicylates, thyroxine, and 2,4-dinitrophenol in high dosage uncouple phosphorylation from oxidation. Uncoupling by thyroxine has been attributed to a change in the spatial arrangement of mitochondrial enzymes as a result of swelling; but this is not the primary action of the hormone, since the mechanism whereby the swelling of mitochondria is produced must still be elucidated. In physiological doses, however, thyroxine causes increased synthesis of nuclear, ribosomal, and cytoplasmic soluble RNA and increases activity of nuclear RNA polymerase and other enzymes involved in nucleic acid and protein synthesis. Hormones, streptomycin and other antibiotics, and the purine and pyrimidine antimetabolites also appear to act by affecting template molecules involved in cell synthetic processes. Since there are a number of steps involved in these processes, many sites of drug action are possible. Thus, insulin is thought to increase the ability of ribosomes to bind and translate messenger RNA, possibly by modification of ribosomal structure, and aldosterone is thought to regulate active sodium transport by an as-yet-unexplained effect of DNA-directed RNA synthesis and *de-novo* synthesis of proteins.

CHARACTERIZATION OF DRUG EFFECTS

The effects of drugs are variously expressed as biochemical or physiological changes, or as the occurrence or relief of clinical symptoms. Thus, drugs are described as anticoagulants, vasoconstrictors, psychotomimetics, analgesics, and so forth. However, for evaluation and comparison of drugs, their effects must be related to dosage, and they must be further characterized in terms of maximum efficacy, variability, and selectivity. Equally important is delineation of the time course of drug effect, both after administration of single doses of the drug and during chronic medication.

Time Course of Drug Effect. The various effects of a drug need not have the same time course. Each effect is commonly characterized by its *latency, time of peak effect,* and *duration.* Although also modified indirectly by the factors that influence duration of effect, the latent period between administration of the drug and onset of effect is determined largely by the route of administration and the rates of absorption and distribution of the drug. The rate of biotransformation to an active metabolite may also be important. Similarly, although also modified by continuing absorption from the site of administration, duration of effect is determined primarily by the rates of inactivation and excretion of the drug. In addition, redistribution from its locus of action to storage sites, physiological reflexes, and development of drug tolerance may also contribute to termination of drug effect. In most cases, as dosage is increased, latency is reduced and duration is prolonged. Duration of effect is also conveniently expressed in terms of the *half-times* for decline of effect and, when it closely parallels drug effect, for decline of plasma concentration of the drug. These indices of duration of effect are particularly useful for describing dosage schedules for chronic medication.

The Dose-Effect Relationship. Ideally, the relationship between dose and effect is based upon the effects attained under equilibrium conditions. However, in practice, the dose-effect relationship is commonly derived from the peak effects after single doses of the drug. There is no single characteristic relationship between intensity of drug effect and drug dosage. A dose-effect curve may be linear, concave upward, concave downward, or sigmoid. Moreover, if the observed effect is the composite of several effects of the drug, such as the change in blood pressure produced by a combination of cardiac, vascular, and reflex effects, the dose-effect curve need not be monotonic. However, a composite dose-effect curve can usually be resolved into simple curves for each of its components; and simple dose-effect curves, whatever their precise shape, can be viewed as having four characterizing parameters: potency, slope, maximum efficacy, and vari-

ability. These are illustrated in Figure 1–3 for the common sigmoid log dose-effect curve. The logarithmic transformation of dosage is often employed for the dose-effect relationship, because it permits display of a wide range of doses on a single graph, and because it has certain mathematical advantages when dose-effect curves are compared.

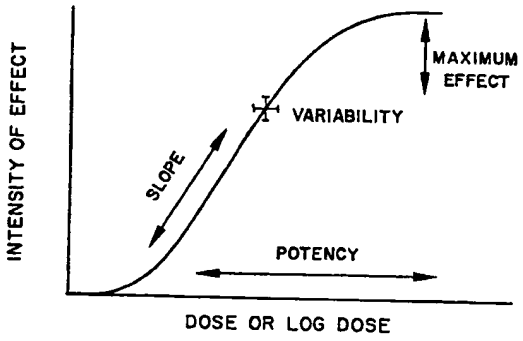


Figure 1–3. *The dose-effect relationship.*

Representative dose-effect curve, illustrating its four characterizing parameters (*see* text for explanation).

Potency. The location of its dose-effect curve along the dose axis is an expression of the potency of a drug. Potency is influenced by the absorption, distribution, biotransformation, and excretion of a drug, as well as being determined by its inherent ability to combine with its receptors. Potency is a relatively unimportant characteristic of a drug, since it makes little difference whether the effective dose of a drug is 1 μg or 100 mg, as long as the drug is administered in appropriate dosage. Potency is not necessarily correlated with any other characteristic of a drug, and there is no justification for the view that the more potent of two drugs is clinically superior. Low potency is a disadvantage only if the effective dose is so large that it is awkward to administer. Extremely potent drugs, particularly if they are volatile or are absorbed through the skin, may be hazardous and may require special handling.

For therapeutic applications, the potency of a drug is necessarily stated in *absolute* dosage units (25 μg, 10 mg/kg, etc.); for comparison of drugs, *relative potency,* the ratio of equieffective doses ($\frac{1}{10}\times$, 5 ×, etc.) is a more convenient expression.

Slope. The slope of the more-or-less linear, central portion of the dose-effect curve is of more theoretical than practical importance. For example, a steep dose-effect curve for a CNS depressant implies that there is a small ratio between the dose that produces coma and that which causes mild sedation, and that excessive or inadequate effect may occur if the dose of the drug is not carefully adjusted. Nevertheless, many factors influence the margin of safety of a drug and the variability of its effects, and these characteristics of a drug are properly expresed by methods that summarize the contributions of all factors (*see* below). It should be noted that doubling the dose of a drug does not necessarily produce twice the effect, and the quantitative relationships between dose and effect should not be confused in this manner.

The significance of the slope and shape of the dose-effect curve in analysis of drug action is beyond the scope of the present discussion. However, it deserves passing mention that mere parallelism of their dose-effect curves is not a reliable basis for concluding that different drugs produce their effects by the same mechanism.

Maximum Efficacy. The maximum effect produced by a drug, even at very large dosage, is termed its *ceiling effect* and is referred to as its maximum efficacy or, simply, efficacy. Maximum efficacy of a drug may be determined by its inherent properties and be reflected as a plateau in the dose-effect curve, but it may also be imposed by other factors. If the undesired effects of a drug limit its dosage, its efficacy will be correspondingly limited, even though it is inherently capable of producing a greater effect. Maximum efficacy of a drug is clearly one of its major characteristics. One of many important differences between morphine and aspirin is that the opiate has sufficient efficacy to provide relief of pain of nearly all intensities, whereas the salicylate is effective only against mild-to-moderate pain.

Efficacy and potency of a drug are not necessarily correlated, and these two characteristics of a drug should not be confused.

Biological Variation. The more important factors that modify drug effect are summarized later in this chapter. However, even when all known sources of variation are controlled or taken into account, drug effects are

never identical in all patients, or even in a given patient on different occasions. A dose-effect curve applies only to a single individual under carefully controlled conditions or to the average individual. The perpendicular brackets in Figure 1–3 indicate that biological variation of the dose-effect relationship can be visualized in either of two ways. The vertical bracket expresses the fact that a range of effects will be produced if a given dose of a drug is administered to a group of individuals; alternatively, the horizontal bracket expresses the fact that a range of doses is required to produce a specified intensity of effect in all individuals.

Dose-Percent Curve. The dose of a drug required to produce a specified intensity of effect in an individual is termed the *individual effective dose.* Individual effective doses of most drugs are lognormally distributed, which means that the familiar *normal curve* of variation is obtained if the logarithms of the individual effective doses for a group of patients are expressed as a frequency distribution. A cumulative frequency distribution of individual effective doses, that is, the percentage of individuals that exhibit the effect plotted as a function of logarithm of dose, is known as a dose-percent curve. Although also a sigmoid curve, the dose-percent curve is not a dose-effect curve but merely an expression of individual variability for a single effect.

The dose of a drug required to produce a specified intensity of effect in 50% of individuals is known as the *median effective dose* and is abbreviated ED50 (*not* ED_{50}). If death is the end point, the median effective dose is termed the *median lethal dose* (LD50). The doses required to produce the stated effect in other percentages of the population are similarly expressed (ED20, LD90, etc.). Similar notations are also often used to refer to the dose of a drug required to produce a stated fraction of the maximum effect or a stated intensity of effect. These conflicting uses of the same abbreviations should not be confused.

Terminology. Specific terms are used to refer to individuals who are unusually sensitive or unusually resistant to a drug, and to describe those in whom a drug produces an unusual effect. If a drug produces its usual effect at unexpectedly low dosage, the individual is said to be *hyperreactive.* Hyperreactivity should not be described as *hypersensitivity,* since this term is usually used to refer to the pattern of effects associated with drug allergy. Hyperreactivity to a drug should be termed *supersensitivity* only if the increased sensitivity is the result of denervation. If a drug produces its usual effect only at unusually large dosage, the individual is said to be *hyporeactive.* Hyporeactivity is also described as *tolerance,* but this term has the connotation of hyporeactivity acquired as the result of prior exposure to the drug. Tolerance that develops rapidly after administration of only a few doses of a drug is termed *tachyphylaxis.* Hyporeactivity should be described as *immunity* only if the acquired tolerance is the result of antibody formation.

An *unusual effect* of a drug, of whatever intensity and irrespective of dosage, that occurs in only a small percentage of individuals is often termed *idiosyncrasy.* However, this term is frequently considered a synonym for drug allergy and has so many other connotations that it probably should be abandoned. Unusual effects of drugs have also been called *meta reactions,* but this term has not gained wide acceptance. Perhaps unusual effects of drugs are best described as such or by terms that refer to the underlying mechanism; they are often types of drug allergy or a consequence of genetic differences.

Selectivity. A drug is usually described by its most prominent effect or by the action thought to be the basis of this effect. However, such descriptions should not obscure the fact that *no drug produces only a single effect.* Morphine is correctly described as an analgesic, but it also suppresses cough and causes sedation, respiratory depression, constipation, bronchiolar constriction, release of histamine, antidiuresis, and a variety of other effects. A drug is adequately characterized only in terms of its full *spectrum of effects.* The relationship between the desired and undesired effects of a drug is termed its *therapeutic index, margin of safety,* or *selectivity.* Rarely is a drug sufficiently selective to be described as being *specific.* For therapeutic applications, selectivity of a drug is clearly one of its more important characteristics.

Because the ideal drug produces its desired effect in all patients without causing toxic effects in any, and because dose-percent curves need not be parallel, it is sometimes argued that therapeutic index should be defined as the ratio between the minimum toxic dose and the maximum effective dose. However, minimum and maximum effective doses cannot be estimated with precision, and therapeutic index is usually defined as the ratio between the median toxic dose and the median effective dose (TD50/ED50). In laboratory studies, therapeutic index is often based upon the median lethal dose (LD50/ED50). In clinical studies, drug selectivity is often expressed indirectly by summarizing the pattern and incidence of adverse effects produced by therapeutic doses of the drug and by indicating the proportion of patients who were forced to decrease drug dosage or discontinue medication because of adverse effects. These indirect procedures are often adequate, but comparison of dose-effect curves for desired and undesired effects is more consistently meaningful and is preferred whenever feasible.

A drug does not have a single therapeutic index, but many. The margin of safety of aspirin for relief of headache is greater than its margin of safety for relief of arthritic pain, since the latter use requires larger dosage. Similarly, several therapeutic indices can be calculated for each desired effect. A synthetic opiate substitute may cause less constipation than morphine and yet afford no advantage over the parent compound with regard to respiratory depression or sedation. Moreover, a drug may be selective within one context yet still be nonselective within another. The antihistamines are correctly described as selective antagonists of histamine, yet none of these drugs produces this selective peripheral effect without also causing significant central sedation. Finally, a drug may be correctly described as having an adequate margin of safety in most patients, but this description is meaningless for the patient who exhibits an unusual response to the drug. Penicillin is essentially nontoxic in the majority of patients, yet it has caused death in those who have become allergic to it.

Biological Assay. Alkaloids and other highly purified drugs obtained from plants and animals can usually be standardized by their chemical or physical properties. However, drugs that are only partially purified, such as certain digitalis preparations and various hormones, vitamins, antibiotics, and vaccines, must be standardized by their biological properties. The estimation of the relative potencies of such preparations by comparison of their biochemical, pharmacological, or toxic effects is termed *biological assay* or, simply, *bioassay*. An effect of the preparation is compared with that of a *reference standard* under carefully controlled conditions. The reference standard is usually a highly purified, often crystalline, preparation of the substance being assayed. The potency of a preparation standardized by biological assay is usually expressed in units, or weight equivalent, of the reference standard. Official drugs have legally required methods of assay, and both the assay procedure and the reference standard are rigidly defined. These are often the same as those specified by an international committee. Nonofficial preparations that are assayed by different methods or against different reference standards may vary considerably in relative potency.

FACTORS THAT MODIFY DRUG EFFECTS AND DRUG DOSAGE

Many factors modify the effects of drugs. Some of these, such as the development of drug allergy, result in *qualitative* differences in the effects of a drug and may preclude its safe use. Others produce only *quantitative* changes in the usual effects of the drug and can be offset by appropriate adjustment of dosage. These variables must be taken into account before a drug is prescribed, and a stated *therapeutic dose* of a drug must be viewed only as the anticipated dose for the average patient or that from which to estimate the dose for an individual patient. Indeed, the optimal dosage for many drugs is determined for each patient only by careful monitoring of drug effect. It is for this reason that it is appropriate to state that *the dose of a drug is "enough."* The physician's legal responsibility if he deviates from dosages recommended in official compendia or in the manufacturer's package insert has been summarized in a Council Conference (1969).

The following are the more important factors that influence the therapeutic dose of a drug.

Body Weight. The ratio between the amount of drug administered and body weight influences the concentrations of the drug at its sites of action. Therefore, drug dosage should be suitably adjusted, particularly for abnormally lean or obese individuals. For adjustment of drug dosage over a wide range of body sizes, body surface area is often a more satisfactory index than is body weight.

Age. Children are often more sensitive than adults to drug-induced changes in water and electrolyte or acid-base metabolism. Special precaution must also be taken in the use of hormones or other drugs that might influence growth and development. Very young infants, particularly premature babies, may be abnormally sensitive to many drugs because of immaturity of renal function or of the enzymatic mechanisms for drug inactivation, or because of incomplete development of the blood-brain barrier. The effects of drugs on the immature brain may also be somewhat unusual.

Although drug dosage for older children may be calculated as a fraction of the adult dose on the basis of body weight or estimated body surface area, that for younger children, and especially for infants, should be learned as such and not calculated by formula. Unfortunately, optimal doses for children have been established for relatively few drugs.

Elderly individuals may respond to drugs in a somewhat abnormal manner, often because of impaired ability to inactivate or excrete drugs, or because of other concurrent pathology.

Sex. Women are thought to be more susceptible than men to the effects of certain drugs, in part because of smaller size. In some instances, this difference is considered sufficient to necessitate reduction in dosage. In addition, the subjective effects of drugs may be somewhat different in the two sexes. During pregnancy, caution is necessary in the administration of drugs that might affect the uterus or fetus. A wise precaution is to avoid the use of all drugs except those essential to maintain pregnancy. Activity of the hepatic microsomal enzymes involved in drug biotransformation is only slightly influenced by sex.

Route of Administration. Inasmuch as rate and extent of absorption and sometimes pattern of biotransformation differ with the route of administration, dosage must be adjusted to take this factor into account. The intravenous dose of a drug is often smaller than the subcutaneous, and this, in turn, is usually smaller than the oral dose.

Time of Administration. The time at which a drug is administered sometimes influences dosage. This is especially true for oral therapy in relation to meals. Absorption proceeds more rapidly if the stomach and upper portion of the intestinal tract are free of food, and an amount of drug that is active before a meal may be ineffective if given after eating. On the other hand, irritating drugs are better tolerated if food is in the stomach. Diurnal and seasonal variations in the effects of drugs are well recognized in animals and may also be important in man.

Rate of Inactivation and Excretion. If function of the organs concerned in inactivation or excretion of a drug is impaired, both intensity and duration of effect of a given dose of a drug may be greater than desired, and toxic effects may occur. Thus, it is of practical importance to know the mechanisms by which a drug is inactivated or eliminated, and to make appropriate adjustment in drug dosage for patients in whom these mechanisms are impaired.

Tolerance. Tolerance may be acquired to the effects of many drugs, especially the opioids, barbiturates and other CNS depressants, nitrites, xanthines, and certain CNS stimulants. When this occurs, *cross-tolerance* may develop to the effects of pharmacologically related drugs, particularly those acting at the same receptor site, and drug dosage must be increased to maintain a given therapeutic effect. Since tolerance does not usually develop equally to all effects of a drug, the therapeutic index often decreases. However, there are also examples of the development of tolerance to the undesired effects of a drug and a resultant increase in its therapeutic index. After tolerance develops, normal sensitivity may be regained only by suspending drug administration. For most drugs, the development of tolerance can be minimized by initiating therapy with the lowest effective dose and by avoiding continuous administration of the drug at regular intervals. In contrast, the emergence of resistant microorganisms during chemotherapy is favored when only minimally effective dosage or intermittent medication is employed.

The mechanisms involved in the development of tolerance are only partially understood. In animals, tolerance often occurs as the result of induced synthesis of the hepatic microsomal enzymes concerned in drug biotransformation; the possible significance of this *drug-disposition tolerance* during chronic medication in man is an area of active investigation. The most important factor in the development of tolerance to the opioids, barbiturates, and ethanol is some type of neuronal adaptation vaguely referred to as *cellular* or *pharmacodynamic tolerance*. Tachyphylaxis, such as that to histamine-releasing agents and to the sympathomimetic amines that act indirectly by releasing norepinephrine, has been attributed to depletion of available mediator, but other mechanisms have also been proposed.

Physiological Variables. Changes in water and electrolyte balance, acid-base status, body temperature, and other physiological factors may also modify the effects of drugs. Unfortunately, no simple summary of the effect of these variables is possible. Metabolic acidosis reduces the saluretic effects of

the carbonic anhydrase inhibitors but increases those of the organic mercurials. Similarly, hypothermia decreases blockade of neuromuscular transmission by tubocurarine but increases that by succinylcholine. Likewise, the effects of drugs may be increased or decreased after denervation.

The effects of drugs, just as those of disease, may be manifest as encroachment upon *physiological reserve* rather than as an overt effect, and this factor must also be considered when drugs are prescribed. Ganglionic blocking agents and other drugs that impair compensatory sympathetic reflexes may have minimal effects upon the blood pressure of a recumbent individual, yet cause orthostatic collapse when he assumes an upright posture. This principle is important in establishing proper dosage of drugs in the therapy of hypertension and in selecting drugs for preanesthetic medication. Similarly, respiratory depression may be manifest primarily as respiratory acidosis with only minimal reduction of rate or depth of breathing or of alveolar ventilation. Failure to appreciate this fact is often responsible for underestimation of the depressant effects of drugs on respiration.

Pathological State. The effects of certain drugs are also considerably modified by pathological conditions. Patients with chronic pulmonary disease or increased intracranial pressure are often unusually sensitive to morphine and other respiratory depressants. Conversely, the hyperthyroid individual can tolerate larger doses of morphine than can the normal person, but he is responsive to a dose of epinephrine that would scarcely affect a person with normal thyroidal function. *Nutritional* deficiencies may also modify the effects of drugs.

Activity of the hepatic microsomal enzymes involved in drug metabolism may be reduced in certain types of hepatic disease. Unfortunately, reduced microsomal activity is not consistently correlated with impaired hepatic function detected by the conventional functional tests.

Milieu. Many drug effects, particularly those on mood and behavior, and the subjective effects of drugs in general, are often susceptible to environmental factors or the "set" imparted at the time of drug administration. The CNS depressants may improve psychomotor performance under one set of experimental or clinical conditions yet impair such performance under other conditions. Similarly, the subjective effects associated with dummy medication in controlled clinical trials vary widely, depending upon the effects of active medication being evaluated concurrently, the manner in which the subjective effects are elicited, and many other factors.

Genetic Factors. Genetic factors contribute to the normal variability of drug effects and are responsible for a number of striking quantitative and qualitative modifications of pharmacological activity. Many of these differences, such as the prolonged apnea in some patients after administration of usual doses of succinylcholine (a neuromuscular blocking agent) have been traced to genetic influences on the concentrations of enzymes involved in *drug biotransformation*. Other variations in drug effect, such as the greater incidence of drug-induced hemolytic anemia in non-Caucasians than in Caucasians, also have been found to be related to genetically determined enzyme patterns that modify the *actions of drugs*.

The objectives of *pharmacogenetics* include not only identification of differences in drug effects that have a genetic basis but also development of simple methods by which susceptible individuals can be recognized *before* the drug is administered.

Drug Interaction. The effects of a drug may be modified by prior or concurrent administration of another drug, and improved therapy is sometimes possible by judicious use of concurrent medication. However, serious adverse effects may also result from drug interaction. Since a patient frequently receives five or more drugs during the course of an illness, the possibility of unplanned combination of drug effects must also be considered. Drug interactions may arise either from alteration of the absorption, distribution, biotransformation, or excretion of one drug by the other, or from combination of their actions or effects.

Multiple-drug therapy is justified if it provides greater efficacy than can be achieved with full doses of single drugs, greater margin of safety, or more satisfactory onset or duration of effect. Sometimes a drug is administered in combination with another to antagonize an untoward effect. However, it is usually more desirable to reduce the dose of the toxic drug or to change medication, rather than to resort to combined medication. Whatever the rationale for multiple-drug therapy, the efficacy and safety of the combined medication must be evaluated in the same manner as for single drugs.

Drug Mixtures. A distinction must be made between the concurrent, but separate, administration of drugs and their administration together as a fixed-dose mixture. Occasionally, as illustrated by the sulfonamide mixtures, the use of fixed-dose mixtures is justified. However, most mixtures of drugs have distinct disadvantages, and their use may involve frank risk. Careful adjustment of dosage is almost always required to attain the maximum benefits from combined medication with a minimum of untoward effects. In some cases, proper timing of drug administration may also be important. The flexibility of dosage and timing essential for the success of combined medication is sacrificed if fixed-dose mixtures are used. In addition, the use of mixtures often complicates therapy, since, if toxic effects occur, it may be impossible to identify the component responsible, and all medication must then be discontinued. The use of mixtures also fosters multiple-drug therapy without first establishing the need for more than one drug. When this occurs, needless expense is imposed upon the patient, and he is unnecessarily exposed to the risk of toxicity from the superfluous components. Additionally, the indiscriminate use of drug mix-

tures fosters careless diagnosis and inappropriate therapy.

Terminology. Descriptions of the combined effects of drugs are often ambiguous, because the terms *addition, summation, synergism,* and *antagonism* are not employed consistently. The usage outlined here is that of Loewe (1953).

Two drugs are said to be *heterergic* for a particular effect if the effect is manifested by one of the drugs but is absent from the spectrum of the other. If the combined effects of heterergic drugs are greater than those of the active component alone, they are said to exhibit *synergism;* if the combined effects are less than those of the active component alone, the interaction is termed *antagonism.* Although often employed as a synonym for synergism, the term *potentiation* should be abandoned, since it has erroneously acquired the connotation of clinical superiority.

Heterergic synergism and antagonism often result from alterations in the distribution, biotransformation, or excretion of the active component. Heterergic antagonism may also involve interaction of the two drugs at the same receptor. If the antagonist acts reversibly, *competitive* antagonism results; if it acts irreversibly, the interaction is termed *nonequilibrium* antagonism. Receptor antagonism is usually *selective* for a given agonist. If the two drugs form an inactive complex, the interaction is termed *chemical* antagonism.

The interaction of drugs that have overtly opposite effects is termed *physiological* or *functional* antagonism; such antagonism is usually *nonselective* and *noncompetitive.*

If two drugs produce the same overt effect, they are termed *homergic.* Descriptions of the combined effects of such drugs are intended to indicate whether they are equal to, or greater or less than, those expected by simple addition. However, two types of additive behavior can be distinguished. If the drugs are close congeners that act on the same receptors (*e.g.,* epinephrine and norepinephrine), *doses* of one drug should substitute for those of the other, in proportion to their relative potency, over a wide range of combinations. Only drugs that exhibit this *dose addition* are properly described as *additive.* Deviations from dose addition are termed *supra-additive* or *infra-additive;* these latter descriptions imply that the drugs act by different mechanisms. If the combined *effects* of homergic drugs are equal to the sum of their individual effects, they are said to exhibit *effect addition* or *summation.* Deviations from summation are usually described as such. Drugs that act by the same mechanism and are additive in all combinations will exhibit summation only in certain combinations. The terms *synergism* and *antagonism* are best avoided for homergic drugs. Whatever terms happen to be used, most descriptions of the combined effects of drugs, particularly in the clinic, refer to effect addition rather than dose addition.

Dosage Schedules for Chronic Medication. To maintain the desired effect without cumulative toxicity during chronic medication, a *maintenance dose* of the drug must be administered at *dosage intervals* that balance the rates of inactivation and excretion. To avoid excessive fluctuation of effect between doses when the drug is rapidly absorbed, maintenance doses must be administered at frequent intervals, perhaps one sixth or less of the half-time for inactivation and excretion of the drug. Alternatively, the drug may be administered by constant intravenous infusion or as a repository preparation that provides slow, continuous absorption over an extended interval. If medication is initiated on a dosage schedule subsequently suitable for maintenance of effect, attainment of the desired effect is necessarily delayed. However, the effect may be achieved more promptly by administration of one or more *priming doses* somewhat larger than the subsequent maintenance doses. Chronic medication is often complicated by the development of drug tolerance and by the fact that medication is frequently interrupted during the hours of sleep. In addition, patients often fail to take medication as consistently and as regularly as directed.

DRUG TOXICITY

No drug is free of toxic effects. They may be trivial, but on occasion they are serious and may be fatal. Some appear promptly, but others may not develop until after prolonged medication. Still others occur only in certain patients or only in combination with other drugs. Some toxic effects of certain drugs, such as hemorrhage during anticoagulant medication, are an extension of the desired effects and can be avoided by proper adjustment of dosage. On the other hand, the desired and undesired effects of a drug may be different manifestations of the same primary action and thus be inseparable. In many instances, an effect of a drug that is sought in one patient becomes an undesired effect in another when the drug is employed for a different purpose. The incidence of untoward effects of drugs varies greatly and unfortunately cannot always be anticipated from animal studies.

Clinicians have long been aware of the drug-induced diseases. However, with the introduction into therapeutic practice of drugs of greater and broader efficacy, the problem of drug toxicity has increased, and it is now considered the most critical aspect of modern therapeutics. Not only is a greater variety of serious toxicity being uncovered, but also the average incidence of adverse effects of medication is increasing and unex-

pected toxic effects are occurring relatively frequently. There is an urgent need for the development of methods in animals that accurately predict the potential harmful effects of drugs in man. There is also need for better procedures for prompt collection, assessment, and dissemination of reports of clinical toxicity. However, adverse effects do not arise solely because of the inherent toxicity of drugs and the limitations of the methods for early detection of this toxicity. Many of the adverse effects could be avoided if drugs were used more carefully and more wisely. The physician should avoid a toxic drug if a less toxic one will suffice; and he should, if possible, avoid the use of concurrent medication and especially the use of drug mixtures, since one drug may affect the toxicity of another. Moreover, he must be aware of the potential hazards of the drugs that he uses, and he must be prepared to act promptly if toxicity occurs. He must be especially alert for the unexpected.

A brief consideration of several of the more serious drug-induced diseases will introduce the reader to this important aspect of pharmacology.

Drug Allergy (Hypersensitivity). Although the incidence of allergic reactions to most drugs is low, the list of known offenders is growing steadily, and drug allergy represents a major problem in the use of some drugs. In addition, there is an urgent need for reliable, safe methods for detecting susceptible individuals before drug administration. The development of such tests is complicated by the fact that metabolites of a drug, perhaps even minor metabolites that are not detected in usual biotransformation studies, as well as the drug itself and even trace impurities may serve as haptens.

Drug allergy may take many forms, including the full spectrum of immediate and delayed types of allergic reactions produced by foreign macromolecules. Skin reactions extend from mild rash to severe exfoliative dermatitis. Those of blood vessels range from acute urticaria and angioedema to severe arteritis with localized medial degeneration. Drug fever is an allergic phenomenon that very closely resembles serum sickness; it is manifested by fever, leukocytosis, arthralgia, and dermatitides. Rhinitis, asthma, and even anaphylactic shock are other familiar allergic responses that can be precipitated by drugs.

Hepatocellular damage, cholestatic jaundice, renal tubular necrosis, depression of hematopoietic functions, photosensitivity, and a number of additional adverse effects of drugs may also be manifestations of drug allergy.

Blood Dyscrasias. Leukopenia, granulocytopenia, aplastic anemia, hemolytic anemia, thrombocytopenia, and, in some cases, defects in the clotting factors are serious, sometimes fatal, complications of drug therapy. Although drug allergy is responsible for many of the cytopenias, certain of the blood dyscrasias are believed to result from a direct toxic effect of drugs on bone marrow. The most common basis for drug-induced hemolytic anemia is a genetically determined deficiency in red-cell glucose-6-phosphate dehydrogenase activity.

Hepatotoxicity and Nephrotoxicity. Because drugs are concentrated in the liver and kidney, damage to these organs by a direct toxic effect of drugs is not uncommon. Hepatotoxicity and nephrotoxicity may also occur as forms of drug allergy. Well-recognized adverse effects on the liver include hepatocellular toxicity, the potentially fatal viral hepatitis–like syndrome produced by the halogenated hydrocarbons and other drugs, and intrahepatic cholestasis, a type of hepatotoxicity that resembles obstructive jaundice and is produced by the phenothiazines, certain steroids, and a number of other agents. In addition, a variety of drugs precipitate hepatic coma in patients with liver disease. Glomerular-tubular damage is an important toxic effect of a number of drugs, including several antibiotics, and intrarenal precipitation of the less soluble sulfonamides is the major cause of the nephrotoxicity of these agents.

Teratogenic Effects. Although the thalidomide tragedy dramatically emphasized that drugs may adversely influence fetal development, there is still little reliable clinical information about the possible teratogenic hazard of most drugs. For this reason, all un-

necessary medication should be avoided during pregnancy. Moreover, since pregnancy is often not diagnosed at the time of greatest vulnerability of the fetus, all drugs not known to be reasonably safe on the basis of long usage should be avoided by women of childbearing age.

Behavioral Toxicity. This term refers to suppression of normal anxiety, reduction in motivation, impairment of memory and learning, distortion of judgment, nonpurposive or inappropriate behavior, and other adverse effects of drugs on mood, behavior, and psychological and psychometric functioning. Motor incoordination and impairment of ability to operate machinery or to drive a motor vehicle may also be considered a form of behavioral toxicity. The term has gained widest use in connection with psychopharmacological agents, but it applies to other drugs as well.

Drug Dependence and Drug Addiction. Any drug that alters mood or behavior is likely to be abused and is potentially capable of producing *drug dependence* upon repeated administration. The drugs that are commonly abused include the opioids; the barbiturates, other sedative-hypnotics, and ethanol; as well as the amphetamines, cocaine, and various other psychopharmacological agents. The characteristics of drug dependence vary with the agent involved. However, one characteristic common to all types of drug dependence is *psychic dependence,* a drive or craving that requires periodic or chronic administration of the drug for pleasure or for relief of discomfort. Another feature of some types of drug dependence is *physical dependence,* a state characterized by the appearance of physical symptoms when administration of the drug is supended. These symptoms are termed the *withdrawal* or *abstinence syndrome. Tolerance* is a characteristic of only certain types of drug dependence.

The term *drug dependence* was coined specifically to permit consideration of the pharmacological and medical aspects of *drug abuse* in isolation from the broader socioeconomic, moral, and legal aspects of the problem that are embraced by the term *drug addiction.* Unfortunately, the two terms continue to be used interchangeably. Abuse of drugs that adversely influence mood and behavior is a problem of increasing medical and social importance. The subject is considered in detail in Chapter 16.

Drug Poisoning. Accidental poisoning is a health problem of major significance. Several thousand deaths from chemical poisoning occur annually in the United States, and it is estimated that the number of nonfatal poisonings exceeds 1 million a year. More than one fourth of these fatalities and about one half of all poisonings occur in children under 5 years of age. The tragedy of this high incidence of poisonings in childhood is that most of them could be avoided.

The physician should assume an active role in *prevention* of poisoning. Parents cannot be too strongly urged to keep drugs out of reach of children, preferably in a "locked" cabinet, and to teach them that medicinals are not candy. The many common household articles that are poisonous should be made unavailable to children, and poisonous pesticides and herbicides should not be placed in the home. Indoctrination against accidental poisoning must also be directed to adults. They should be urged to read and heed labels on medicines, and they should be encouraged to discard residual prescription drugs that are no longer needed. Accidental poisoning among adults is not uncommon and usually results either from attempts at self-medication or from mistaking one drug for another.

The number and variety of drugs and chemicals that might be encountered in poisonings are enormous. However, the physician should become familiar with the details of treatment for the more commonly encountered household poisons. He should also be prepared to treat intoxication caused by agents frequently used for suicidal purposes, such as the barbiturates, other CNS depressants, and carbon monoxide, and to detect and treat those types of industrial intoxications that might occur in his community. Additionally, he should take advantage of the services and information available to him through his regional Poison Control Center.

Diagnosis of Drug Poisoning. The diagnosis of poisoning may be difficult, since there is scarcely a syndrome produced by a toxic agent that cannot simulate disease. However, there are a number of helpful signs and symptoms that assist in the detection of acute poisoning, particularly if this possibility is included in the differential diagnosis. In this connection, contaminated foods are often the etiological agent. The onset of symptoms is usually sudden and follows the taking of food, drink, or medicine by an individual who has been previously well. In some cases, several individuals may suffer from similar symptoms after partaking of the same food. In addition, many drugs leave a telltale odor, produce irritation of the mucous membranes of the mouth and throat, or cause typical pharmacological effects. As a final check, there are often simple chemical tests that can be performed to confirm the diagnosis.

The diagnosis of chronic poisoning is often more difficult. At times, the symptoms and signs are not sufficiently characteristic to point to the toxic agent, and laboratory procedures may be essential. In many cases, only painstaking probing of the patient's history, habits, daily activities, and working conditions leads to the diagnosis. The more uncommon types of industrial poisoning may go undiagnosed until irreparable injury has been suffered by the patient.

Treatment of Drug Poisoning. Most emergency therapy of drug poisoning is symptomatic, since success often depends upon celerity, and valuable time cannot be wasted in attempts at a positive identification of the specific cause of the patient's illness. However, therapy is facilitated if the responsible agent and the degree of exposure or the amount ingested can be determined. The usual steps taken are (1) supportive or symptomatic therapy, (2) termination of exposure and removal of the poison from the body, and (3) administration of antidotes.

Adequate *supportive therapy* is the most important aspect of the treatment of drug poisoning. Serial measurement and charting of the vital signs and important reflexes are helpful procedures by which to judge the progress of the intoxication, the response to therapy, and the need for additional treatment. The patient should be kept in bed and warm, under competent surveillance, and particular attention should be paid to the *respiration, circulation, hepatic and renal function,* and *acid-base and fluid-and-electrolyte balance.* This usually necessitates hospitalization.

Poisons that have been applied externally are best removed by copious *washing* with water, or with a suitable organic solvent if the poison is not water soluble. Induced vomiting, gastric lavage, and oral administration of *activated charcoal* may be employed to reduce further absorption of an ingested poison. The relative merits of these procedures have been reviewed by Gosselin and Smith (1966). Vomiting and gastric lavage are contraindicated in the treatment of poisoning by corrosive agents, strychnine, other convulsants, kerosene, or other hydrocarbon solvents.

Much emphasis has been placed on the use of *emetics,* since vomiting may be as productive as gastric lavage and can be instituted more promptly. However, such drugs are contraindicated in the unconscious patient, and they may not be effective if the patient has ingested a CNS depressant. *Ipecac syrup* is orally effective, but vomiting may not occur for 20 to 30 minutes. It is not dependable if the patient has previously received activated charcoal. *Apomorphine* acts more promptly and certainly, but it must be administered parenterally. It may also cause protracted emesis and CNS depression. Powdered mustard, administered orally as a suspension in warm water, is a household article that can be administered in an emergency. Mechanical stimulation of the oropharynx is unreliable and relatively ineffective.

Gastric lavage, when performed by a trained person, is an effective method for evacuation of the stomach. However, the procedure is time consuming and may not be reliable if the poison is insoluble. Care must be taken to prevent aspiration of gastric contents or lavage fluid into the lungs. After the stomach has been thoroughly emptied, a saline cathartic is sometimes administered through the stomach tube to diminish further intestinal absorption of the poison.

Activated charcoal, administered orally as a fine powder suspended in water, adsorbs a wide variety of chemicals (but not cyanide) and is an effective procedure for retarding absorption of ingested poisons. Since the combination of drug and charcoal is usually reversible, the gastric contents should be removed by lavage or by apomorphine-induced vomiting. The so-called universal antidote, a mixture of activated charcoal, magnesium oxide, and tannic acid, should be abandoned, since it is less effective than activated charcoal alone. Household articles that are somewhat useful for dilution or adsorption of poisons are milk, beaten egg white, flour and starch. Burnt toast is ineffective.

In some instances, the renal *excretion* of a drug can be increased by administration of an osmotic diuretic and by appropriate acidification or alkalinization of the urine. Volatile substances that are excreted by the lungs can often be more rapidly eliminated by stimulating respiration, and the inhalation of carbon dioxide is sometimes employed for this purpose. In cases of severe drug intoxication, elimination of the drug by *peritoneal dialysis* or by use of the *artificial kidney* is a highly effective procedure.

Antidotes serve only a limited role in the treatment of drug poisoning, even when the poison has been identified, because there are safe, effective, selective antagonists for relatively few drugs. Those that are available for individual poisons and drugs are discussed throughout the text. Outstanding examples are the use of chelating agents in metal poisoning, nitrites and thiosulfate in cyanide intoxication, methylene blue in methemoglobinemia, and atropine and reactivators of acetylcholinesterase in poisoning by insecticides and other drugs that inhibit the enzyme.

DEVELOPMENT, EVALUATION, AND CONTROL OF DRUGS

The average practitioner is not usually directly concerned in the evaluation of drugs. Nevertheless, he must know something about the development and evaluation of therapeutic agents, since this knowledge has an important bearing on his attitude toward new drugs and their use. The physician must also be familiar with the laws that regulate the use of medicinals.

Sources and Discovery of New Drugs. The earliest medicinals were crude powders, juices, or extracts from animal, plant, and mineral sources, and these continued to be the only drugs until early in the nineteenth century. However, once advances in chemistry permitted the isolation, purification, and identification of the active constituents of these substances, it was just a step to structural modifications of existing drugs, the synthesis of new ones, and the beginning of the modern age of pharmacotherapy.

New therapeutic agents are discovered by screening, structural modification of established drugs, or accident. Screening refers to the testing of random compounds for selected types of pharmacological activity. The testing of thousands of soil samples for antibiotic activity is an example of this time-consuming, somewhat unsophisticated, relatively inefficient approach. However, it has the important advantage that it can uncover valuable new chemical classes of drugs.

Structural modifications of an established drug often yield congeners that differ only insignificantly from the parent and are aptly termed "me-too" drugs, the marketing of which is hard to justify. However, as discussed in the section on structure-activity relationship above, such alterations do often yield a congener with pharmacological properties significantly different from those of the parent compound. For example, the first three sulfonamides introduced as chemotherapeutic agents, a milestone in the history of drug therapy, are now obsolete. Another particularly fruitful approach has been the structural modification of endogenous molecules involved in cellular metabolism. This has led to the discovery of useful antimetabolites. Rapid advances in the elucidation of basic cellular function make it likely that more and more drugs of this type will be developed in the future.

Many significant advances in therapy in the past have resulted from fortuitous discovery of new drugs or new uses of established therapeutic agents by alert observation in the laboratory or clinic. Well-known examples of this approach are the discovery of penicillin by Fleming and the recognition of the diuretic activity of organic mercurials employed in the treatment of syphilis. Undesired effects of older agents have also been exploited in the development of new drugs, as illustrated by the evolution of the carbonic anhydrase inhibitors, the antihypertensive thiazides, and the oral hypoglycemics from the antibacterial sulfonamides.

Development and Evaluation of New Drugs. The development and evaluation of new drugs in the United States is rigidly controlled by federal regulations administered by the Food and Drug Administration (*see* below). A new drug may not be marketed for general clinical use until it has been subjected to thorough clinical pharmacological studies, and until "substantial evidence" of its efficacy and safety has been obtained from adequate, well-controlled clinical trials conducted by qualified investigators.

Before initial studies in man are permitted, the full pharmacological spectrum of a new drug must be thoroughly and extensively explored in *animals*, and both acute and chronic toxicity tests must be conducted on several species. Because of species variations, such studies are considered useful merely as evidence that the drug has sufficient promise and is sufficiently safe to warrant testing in man. Even the most extensive studies in animals cannot substitute for successful clinical trials as evidence of clinical efficacy. The *initial clinical trials* of a new drug are necessarily cautious experiments, on volunteer normal subjects as well as patients, aimed primarily at establishing that the drug merits further study. If the initial trials in patients provide promise of clinical efficacy, the drug is subjected to thorough *clinical pharmacological studies*, and documentation of its efficacy and safety is sought in *controlled*

clinical trials. Only under exceptional circumstances may a new drug be administered to an individual without his informed consent.

What constitutes an adequately controlled clinical trial necessarily varies, depending upon the drug effect being evaluated. The more important general requirements for all trials are an appropriate and *sensitive method* of evaluation, an *adequate number of subjects, lack of bias, concurrent comparison* of the new drug with a *reference drug* over a *range of doses,* and appropriate *statistical validation.* Many clinical trials must be conducted under so-called *blind* conditions. In a blind experiment, the nature of the medication is concealed from the patient (single-blind) or from both the patient and all persons associated with conduct and evaluation of the trial (double-blind). Blind conditions are particularly essential for trials in which subjective effects of medication are being studied; they may also be necessary in evaluation of certain objective drug effects, if these are under voluntary control or otherwise easily biased. In addition to being compared with a reference drug, a new compound is often compared with inert *dummy* medication, to serve as a control for *placebo effects,* namely, those that are temporally correlated with administration of a drug but cannot be attributed to its pharmacological properties. Placebo effects result in part from the significance of the therapeutic effort to the patient and the set imparted at the time of drug administration. However, other coincident effects, such as spontaneous remission of symptoms, also contribute to the value of a placebo control.

In certain limited circumstances, a *placebo* may be administered in the course of regular therapeutic practice. If an inert lactose capsule or an injection of saline solution is employed for this purpose, it is termed a *pure placebo.* If a subeffective dose of a vitamin or other active drug is used, it is termed an *impure placebo,* since the resulting placebo effect may be erroneously attributed to pharmacological properties of the drug. The administration of a placebo in clinical practice is *not* a reliable procedure by which to distinguish between "psychogenic" and "somatic" disorders on the basis of whether relief of symptoms is obtained.

Because the capacity of existing facilities for conducting controlled clinical trials is limited, and for other practical reasons, controlled trials cannot possibly be conducted on all types of patients or under all varieties of clinical conditions. Consequently, evaluation of the efficacy of a drug may continue well into the period of its *general clinical use.* More important, since drug toxicity may occur only in a limited portion of the population, only after long chronic use, or only in combination with other variables, accurate assessment of the toxic potential of a new drug may not be possible until it has been in general use for several years.

Drug Regulations. There are a number of regulations and compendia concerned with the testing, labeling, purity, and quality of foods, drugs, and cosmetics. Such regulations are designed for the protection of the public health. The following regulations apply to the United States; other countries have similar codes.

Food and Drug Administration. The Federal Food, Drug, and Cosmetic Act of 1938 assures the quality and purity of drugs, by requiring accurate labeling of all medicinals. The initial law, the Federal Pure Food and Drugs Act, was passed by Congress in 1906, as a result of the excessive adulteration and misbrandings of foods and drugs existing at that time. The subsequent modifications of the act—in 1938 and 1962—were, unfortunately, a result of tragedies brought about by inadequate testing of new drugs or vehicles for drugs. Enforcement of the law is entrusted to the Food and Drug Administration (FDA) of the Department of Health, Education, and Welfare. Regulations resulting from the Drug Amendments Act of 1962 are concerned with establishing the efficacy as well as the safety of new drugs. In addition, they place responsibility with the FDA for continuing evaluation of the toxicity of drugs already in general use. Drugs found to be too dangerous in proportion to their therapeutic worth can be removed from the market. Another provision of the Drug Amendments Act was a retro-

spective evaluation of the efficacy of all drugs introduced from 1938 to 1962. This important and difficult review process was undertaken for the FDA by panels of experts organized by the Drug Research Board of the National Academy of Sciences–National Research Council.

Because of severe abuses of barbiturates, psychopharmacological agents, and other centrally acting drugs, the Comprehensive Drug Abuse Prevention and Control Act was passed in 1970. This gives the Bureau of Narcotics and Dangerous Drugs, Department of Justice, special powers to regulate the distribution of drugs liable to be abused.

Decisions as to whether drugs may be sold "over the counter" or dispensed only on prescription and the refilling of prescriptions are regulated by the FDA under the provisions incorporated in the Durham-Humphrey Amendment of 1952 (*see* Appendix). The FDA establishes special standards for insulin, antibiotics, and germicides and is responsible for certification of their safety and efficacy. The safety of food additives and the purity and quality of foods and cosmetics are also under the jurisdiction of the FDA. The purity and the efficacy of veterinary preparations are controlled as rigidly as are human medicinals.

The United States Pharmacopeia (U.S.P.) and The National Formulary (N.F.). The Federal Pure Food and Drugs Act of 1906 recognized the U.S.P. and the N.F. as "official compendia," thereby giving official status to the drugs and the standards set forth in these volumes. The approved therapeutic agents used in medical practice are described and defined with respect to source, chemistry, physical properties, tests for identity, tests for purity, assay, method of storage, and average therapeutic dosage. They were initially written as guides to the physician in his choice of drugs. However, because they serve as official standards for the quality and purity of drugs, these compendia are now of more use to the pharmaceutical industry and the FDA than to physicians. Nevertheless, the prescribing of *official* drugs listed in either the U.S.P. or N.F. provides assurance to the physician that the patient will receive exactly what has been prescribed with respect to quality and chemical uniformity. However, no tests of biological equivalence of official drugs are required, but they are under serious consideration. Other nations have similar compendia; there is also a *Pharmacopoea Internationalis* (sponsored by WHO), as well as a *European Pharmacopoeia* (1st ed., 1970).

U.S.P. Most of the preparations in the U.S.P. are single drugs. In the case of those that must be compounded, instructions are given for their preparation. The U.S.P. organization also provides reference standards for the assaying and testing of many of the U.S.P. drugs.

The first *Pharmacopeia* in the United States was published in 1820, and since that time numerous revisions have appeared. The current edition, U.S.P. XVIII, became official in 1970. The *Pharmacopeia* is revised at five-year intervals. The U.S.P. is revised by a special Pharmacopeial Committee, the members of which donate their services in the interest of the important function they serve. The committee consists of outstanding pharmacologists, physicians, and pharmacists.

N.F. This compendium was first published in 1888 under the name *National Formulary of Unofficial Preparations*. After acceptance as an official drug standard in 1906, the name was changed to its present title. The current edition is N.F. XIII, published in 1970.

The N.F. formerly contained drugs on the basis of demand as well as of therapeutic value, but beginning with N.F. XII therapeutic value was adopted as the sole criterion for the admission of drugs. Many drugs that have been deleted from the U.S.P. appear in the N.F. Unlike the U.S.P., it also contains formulas of certain drug mixtures. The N.F. is published by the American Pharmaceutical Association. It is prepared by a committee and advisory panels consisting mainly of outstanding pharmaceutical scientists and physicians.

Other Regulative Laws. The laws embodied in the *Harrison Narcotic Act* and the *Federal Marihuana Regulations* are superseded by the Comprehensive Drug Abuse Prevention and Control Act of 1970 and are enforced by the Bureau of Narcotics and Dangerous Drugs, U.S. Department of Justice; this act also includes other drugs subject to abuse. The law controls the distribution of opium, coca, cannabis, and any of their natural or synthetic derivatives, barbiturates, amphetamines, LSD, etc. (*see* Appendix). In addition, state and city laws exist to regulate the sale of narcotics, barbiturates, and similar drugs.

GUIDE TO THE "THERAPEUTIC JUNGLE"

The flood of new drugs in recent years has provided many dramatic improvements in therapy, but it has also created a number of problems of equal magnitude. Not the least of these is that of the "therapeutic jungle," the term used to refer to the combination of the overwhelming number of drugs, the confusion over nomenclature, and the associated uncertainty of the status of many of these drugs. A reduction in the marketing of close congeners and drug mixtures and an improvement in the quality of advertising are important ingredients in the remedy for the "therapeutic jungle." However, the physician can also contribute to the remedy by adopting a "way of thinking about drugs" based upon general pharmacological principles, by employing nonproprietary rather than proprietary names whenever possible, by using prototypes both as an instructional device and in clinical practice, by adopting a properly critical attitude toward new drugs, and by knowing and making use of reliable sources of information.

Drug Nomenclature. The existence of many names for each drug, even when reduced to a minimum, has led to a lamentable and confusing situation in drug nomenclature. In addition to its formal *chemical* name, a new drug is usually assigned a *code* name by the pharmaceutical manufacturer. If the drug appears promising, and the manufacturer wishes to place it on the market, a *United States Adopted Name* (USAN) is selected by the USAN Council, which is jointly sponsored by the American Medical Association, the American Pharmaceutical Association, and the United States Pharmacopeial Convention, Inc. (*see* Appendix). This *nonproprietary* name is often referred to as the *generic* name, but this latter term is properly reserved to designate a chemical or pharmacological class of drugs, such as sulfonamides or sympathomimetics. If the drug is eventually admitted to the U.S.P. or N.F., the USAN becomes the *official* name. However, the nonproprietary name and the official name of an older drug may differ. There is increasing worldwide adoption of the same name for each therapeutic substance. For newer drugs, the USAN is usually adopted for the nonproprietary name in other countries, but this is not true for older drugs. International agreement on the USAN name is mediated through the World Health Organization and the pertinent health agencies of the cooperating countries. Subsequently, the drug will also be assigned a *proprietary* name or *trademark* by the manufacturer. If the drug is marketed by more than one company, it may have several proprietary names. If mixtures of the drug with other agents are marketed, each such mixture may also have a separate proprietary name.

To minimize confusion, the nonproprietary or official name of a drug should be used whenever possible, and this practice is adopted in this textbook. The question arises, however, whether the nonproprietary name or a proprietary name should be used by the physician when prescribing drugs. The use of the nonproprietary name is clearly less confusing when the drug is available under multiple proprietary names. However, an important problem concerns the distinction between chemical, biological, and clinical equivalence of drugs. *Chemical equivalents* are identical dosage forms that contain identical amounts of the same chemical substance and meet the physicochemical standards in the official compendia. *Biological equivalents* are those chemical equivalants that, when administered in the same amounts, provide the same biological or physiological availability, as measured by plasma or tissue levels. *Clinical equivalents* are those chemical equivalents that, when administered in the same amounts, provide the same therapeutic effects, as measured by the control of a symptom or a disease. The clinical equivalence of commercial products has been the center of heated controversy.

The main question has been whether drugs that are chemical equivalents are, in fact, clinical equivalents. This is important, since drugs marketed by different companies under the nonproprietary name may differ markedly in cost from the same drug marketed under the proprietary name. Since clinical equivalence of drugs is difficult to quantify because of marked individual differences and of vari-

ations in the symptoms or disease being studied in man, attention has been directed to assessment of the biological equivalence of drugs. This is based on the sound premise that equal tissue or plasma levels of two different preparations of the same drug will give the same therapeutic effect. Tests of biological equivalence of preparations from various companies have demonstrated that differences do occur with drugs that are administered orally in solid dosage forms, such as tablets and capsules. These differences are due to variations in formulation factors, such as particle size, crystal form, adjuvants, fillers, binders, and pressures used to compress tablets, all of which affect the dissolution rate, an important factor in oral absorption of drugs. Even preparations of the same drug with dissolution rates within official standards may not be biological equivalents. Unfortunately, since the majority of medicinals have not been evaluated for biological equivalence, the magnitude of the problem cannot be stated. However, it would be expected to be much less important for highly soluble drugs and even for poorly soluble drugs that are formulated under well-controlled conditions and that meet rigid standards. Until biological and clinical equivalence of medicinals can be assured, the physician is well advised to prescribe only preparations of high quality. *It is, therefore, recommended that drugs be prescribed by nonproprietary name in all cases. However, when a particular dosage form or special formulation of a drug is desired, and especially when accurate control of dosage is critical, the prescription order should then include, in addition to the nonproprietary name, the name of the manufacturer of the preparation desired* (*see* Appendix).

Use of Prototypes. For teaching purposes, as illustrated in this textbook, the confusion created by the welter of similar drugs is reduced by restricting major attention to prototypes in each pharmacological class. Focusing on the representative drugs results in better characterization of a class as a whole, and thereby permits sharper recognition of the occasional member that possesses unique properties. A teaching prototype is often the agent of choice, but this is not always true. A particular drug may be retained as the prototype, even though a new congener is clinically superior, either because more is known about the older drug or because it is more illustrative for the entire class of agents.

The clinician will also find the prototype device helpful in his struggle with the surfeit of congeneric drugs, since his needs for therapeutic agents can usually be adequately satisfied by one or two drugs in each class. Which of a number of more-or-less equivalent drugs the physician actually chooses as his prototypes may be determined by differences in their duration of action or other secondary characteristics. The important consideration is that he restrict his attention to a limited number of drugs in each class and that he become thoroughly familiar with their individual characteristics. If he does, he will inevitably use these agents more effectively than he would if he were to change repeatedly among a larger number of drugs. Moreover, the greater experience with a few drugs will provide a better base line of personal experience by which he may judge the claims for newer medicinals.

Attitude toward New Drugs. A reasonable attitude toward new drugs is summarized by the adage that advises the physician to be "neither the first to use a new drug nor the last to discard the old." This advice is intended as a reminder that only a minor fraction of the new drugs released each year represents significant therapeutic advance, and that the efficacy and safety of a new drug, particularly relative to older agents, may not be fully assessed until sometime after it has been in general clinical use. It also stresses the physician's obligation to keep abreast of significant advances in pharmacotherapy. However, appropriate, timely change from the old drug to the new is possible only if the physician has access to prompt, unbiased, critical information about new drugs.

Information about Drugs. *Pharmacology textbooks* usually provide basic pharmacological principles, critical appraisal of the therapeutically useful classes of drugs, and

detailed descriptions of the prototypes that serve as standards of reference for assessing new drugs. However, for obvious reasons, these textbooks cannot include information on the most recently introduced drugs, nor can they provide detailed descriptions of many of the older drugs. *Remington's Pharmaceutical Sciences,* the *United States Dispensatory and Physicians' Pharmacology,* the *American Hospital Formulary Service,* and various drug encyclopedias are sources of information about drugs; historical accounts of older drugs are found in the older editions. The *Physicians' Desk Reference* (*PDR*) is a convenient source of information about available products, dosage forms, contraindications, untoward reactions, and allowable therapeutic claims, but it is *not* useful as a critical guide to therapy or as a source of pharmacological effects of drugs. Legally, all information on drugs described in *PDR* must conform to the descriptive material in the package insert. The information, in turn, must meet the approval of the FDA. The *American Drug Index* is also a useful cross-indexed source of drug products and dosage forms, listed by both nonproperietary and proprietary names.

Somewhat more current information about drugs is provided by *AMA Drug Evaluations* (*ADE*), a publication of the Council on Drugs of the American Medical Association. The Council previously published several annual indices of drugs, which were sequentially named *New and Nonofficial Remedies, New and Nonofficial Drugs,* and *New Drugs. ADE* is a useful and authoritative reference book that evaluates information on both old and new single-entity drugs and mixtures, arranged according to therapeutic category. It is designed to help the physician in the selection and use of drugs. A very useful source of information on new drugs is the biweekly publication, *The Medical Letter on Drugs and Therapeutics.* Its distinguished board of editors provide a distinct service to medicine and to physicians by furnishing prompt, unbiased, pointed assessment of new drugs. A British publication, the *Drug and Therapeutics Bulletin,* is also published fortnightly with the same high standards. The *Prescribers' Journal,* published bimonthly by the British Ministry of Health, also contains objective and critical analyses of new therapeutic agents. *Pharmacology for Physicians* is a monthly publication in which experts discuss the clinical pharmacology of drugs used for the therapy of particular diseases. Other current and critical sources of information on drugs, drug therapy, and drug toxicity include the periodic appraisals sponsored by the Council on Drugs and published in the *Journal of the American Medical Association,* and the editorials and short reviews in *Clinical Pharmacology and Therapeutics,* the *New England Journal of Medicine,* and similar periodicals. *Pharmacological Reviews* and *Annual Review of Pharmacology* are excellent sources of basic pharmacological information.

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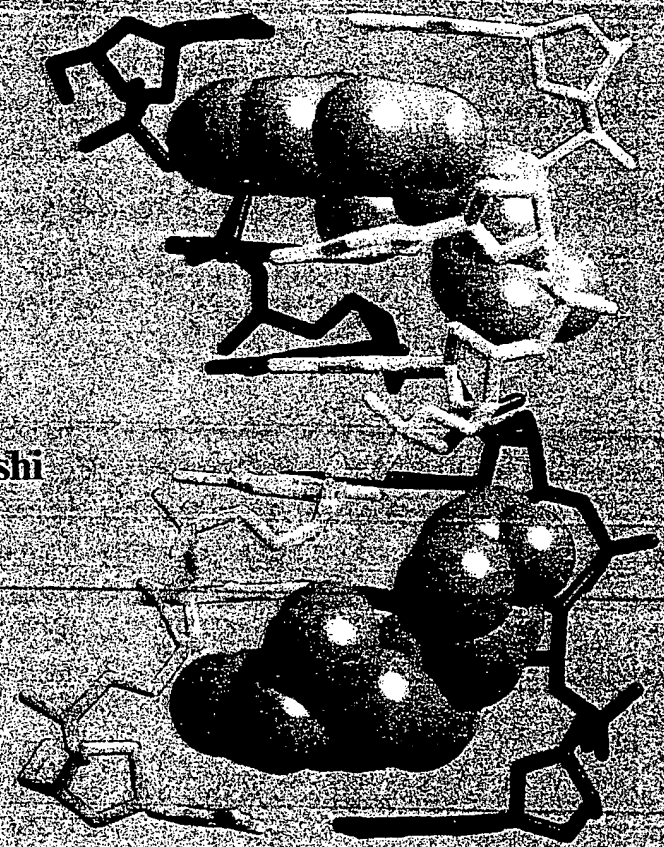

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In vitro and *in vivo* pharmacologic activities of antisense oligonucleotides

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Summary: The use of antisense oligonucleotide as pharmacologic agents is a derivative of the central dogma of molecular biology and knowledge of the physical and chemical properties that govern the structure of nucleic acids. Oligonucleotides have been reported to inhibit the growth of a large number of viruses in cell culture, as well as the expression of numerous ocogenes, a variety of normal genes and transfected reporter genes controlled by several regulatory elements. The therapeutic activity of antisense compounds in animal disease models have also been reported.

This review provides some general conclusions and trends regarding the pharmacologic action of antisense oligonucleotides, that can be formulated from studies previously reported in the literature. In addition, data is highlighted for two specific examples in which antisense oligonucleotides have demonstrated activity against herpes viruses and intracellular adhesion molecule RNA targets.

Introduction

In the past few years, many papers have been published demonstrating the activity of numerous antisense oligonucleotides, of different sequences and chemical type, in a variety of cell-based systems. Recently there have been a number of excellent reviews that have summarized the activities of these compounds in detail (Cohn, 1989; Uhlman & Peyman, 1990; Cazenave & Helene, 1991). As such this review will not attempt to duplicate those comprehensive efforts; instead it will provide a brief summary of the activities of oligonucleotides in cell-based assays and attempt to provide some general conclusions and trends that can be formulated from these previously published data. In addition, this paper will provide examples of data compiled in our laboratories that relate to the pharmacological activities of phosphorothioate oligonucleotides directed against cellular and infectious disease targets.

Pharmacological activities in cell-based models

Oligonucleotides have been reported to inhibit the growth of a large number of viruses in cell culture, as well as the expression of numerous oncogenes, a variety of normal genes and transfected reporter genes controlled by several regulatory elements. These studies varied in the types of oligonucleotides used, the cells used, the RNAs and specific receptor sequences targeted and the conditions employed. Although a wide range of oligonucleotide concentrations have been used to treat cells, only a few studies have reported detailed dose-response curves and clearly documented the purity of the oligonucleotides used. Table I summarizes the information from more than 40 papers in which oligonucleotides were tested for pharmacologic

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Table I Summary of cell-based *in vitro* activities of antisense oligonucleotides

Target viruses	*Cell type*	*Serum*	*Oligo types*	*Oligo length*	*Effective oligo concentration*	*References*
HTLV-III	H9 cells	–	P	12–26	5–50 mg/ml	Zamecnik *et al.* [1986]
HIV	H-T cells	+	P-S	14–28	0.5 μM	Matsukura *et al.* [1987]
HIV (gag/pol)	H-T cells	+	P-S	18–24	1–10 μM	Kinchington & Galpin [1989]
HIV	H9 cells	+	PS, others	20	4–20 μg/ml	Agrawal *et al.* [1988]
HIV	CZM cells	+	PS	18–28	10 μM	Vickers *et al.* [1991]
Herpes simplex	Vero cells	+	CH3-P	7	50–100 μM	Smith *et al.* [1989]
Herpes simplex	HeLa cells	+	PS	28	1–10 μM (non-antisense)	Gao *et al.* [1989]
Herpes simplex	Vero cells	+	CH3-P	12	20–50 μM	Kulka *et al.* [1989]
Herpes simplex	Vero cells	+	CH3-P-psoralen	12	5 μM	Kulka *et al.* [1989]
Herpes simplex	HeLa cells	+	PS	21	0.2–4 μM	Draper & Brown-Driver [1991]
Vesicular stomatitis	L929 cells	+	CH3-P	9	25–50 μM	Agris *et al.* [1986]
Vesicular stomatitis	L929 cells	+	P-lipid	11	50–150 μM	Shea *et al.* [1991]
Vesicular stomatitis	L929 cells	+	p-poly l-lysine	10–15	0.1 μM	LeMaite *et al.* [1987]
Influenza	MDCK cells	+	P-acridine	11	50 μM	Zerial *et al.* [1987]
Tick borne encephalitis	–	+	Various	Various	0.1–1 μM	Vlassov [1989]
SV40	MDCK cells	+	CH3-P	6–9	25 μM	Miller *et al.* [1985]
Rous	Chicken fibroblasts	+	Various	Various	10 μM	Zamecnik & Stephenson [1978]
Hepatitis B	Alexander	+	P	15	8.5 μM	Goodarzi *et al.* [1990]
Bovine papilloma virus	C-127 cells	+	PS	4–30	0.01–1 μM	Cowsert & Fox [1991]

Oncogenes						
c-myc	T-lymphocytes	+	P	15	30 μM	Heikkila *et al.* [1987]
c-myc	HL-60 cells	+	P, PS	15	10 μM	Wickstrom *et al.* [1989]
c-myc	Burkitt cells	−	P	21	100 μM	McMananway *et al.* [1990]
c-myb	PMBC	+	P	18	40 mg/ml	Gewirtz *et al.* [1989]
BCL-2	L697 cells	−	P ± PS	20	25–150 μM	Reed *et al.* [1990]
N-ras	T15 cells	+	CH3-P	9	Inactive	Tidd *et al.* [1988]
Host genes						
Multiple drug resistance	MCF-1 cells	+	PS	15	—	Jaroszewski *et al.* [1990]
PCNA (cyclin)	3T3	+	P	18	30 μM	Jaskulski *et al.* [1989]
Prothymosin	Human myeloma cells	−	P	16–21	40 μM	Sburlati *et al.* [1991]
T cell receptor	T cells	+	P	22	—	Zheng *et al.* [1989]
Gm CSF	Endothelial cells	−	P	15, 18	10^{-5} M	Zheng *et al.* [1989]
CSF-1	FL-ras/myc cells	+	P	—	—	Birchenall *et al.* [1989]
EGF receptor		+	P	13	30 μM	Yeoman *et al.* [1989]
β Globin	Rabbit reticulocytes	+	CH3P	9	100 μM	Blake *et al.* [1985]
cAMP-protein kinase II_β	HL-60	+	P	21	15 μM	Tortora *et al.* [1990]
Phospholipase A2 Activating protein	BC3H1	+	P	25	25 μM	Clark *et al.* [1991]
bFGF	Human astrocytes	−	P	15	10–75 μM	Morrison [1991]
TAU	Neurons	−	P	20–25	3–50 μM	Caceres & Kosik [1990]
Myeloblastin	HL-60 cells	+	P	18	—	Bories *et al.* [1989]
ICAM-1	A549, HUVEC	−	PS	18–20	0.01–1 μM	Chiang *et al.* [1991]
IL-2	T lymphocytes	−	P	15	5 μM	Harel-Bellan *et al.* [1988]
IL-1α	HUVEC	+	P	18	10 μM	Maier *et al.* [1990]
IL-1β	Monocytes	+	PS	15	0.1–2.5 μM	Manson *et al.* [1990]
IGF-1	Myoblasts	−	P	15	10 μM	Florini & Ewton [1990]
Perforin	T lymphocytes	−	P	18	5–35 μM	Acha-Orbea *et al.* [1990]

Table I (*contd.*)

Target	*Cell type*	*Serum*	*Oligo types*	*Oligo length*	*Effective oligo concentration*	*References*
Other						
Chloramphenicol acetyl transferase	CV-1 cells	+	P, PS, CH3P	21	5–30 μM	Marcus-Sekura *et al.* [1987]
Placental alkaline Phosphatase driven by HIV TAR	SK-mel-2 cells	+	PS	18–28	0.25–5 μM	Vickers *et al.* [1991]
Chloramphenicol acetyl transferase driven by human papilloma virus E2 responsive element	C-127 and CV-1 cells	+	PS	14–20	1–10 μM	Cowsert & Fox [1991]

cAMP = cyclic AMP; EGF = epidermal growth factor; G-CSF = granulocyte colony-stimulating factor; GM-CSF = granulocyte macrophage colony-stimulating factor; HB = hepatitis B; HIV = human immunodeficiency virus; HSV = herpes simplex virus; HTLV = human T cell lymphotrophic virus; IV = influenza virus; PCNA = proliferating cell nuclear antigen; RSV = Rous sarcoma virus; TAR = TAT response element; TBE = tick-borne encephalitis; VSV = vesicular stomatitis

activities against a variety of viruses, oncogenes, host genes and transfected reporter genes.

The data presented in Table I support only a few generalizations. First, while phosphodiesters are rapidly degraded in biological systems, a number of investigators have reported activities for unmodified phosphodiester oligonucleotides in cells incubated in the absence of serum or in medium supplemented with heat-inactivated serum. When phosphodiester oligonucleotides have displayed activity, concentrations of more than 10 μM were required. The explanation for these activities is unclear. Considering the presence of endo- and exonucleases that are found within cells it is reasonable to think that these oligonucleotides would be degraded in the cell very rapidly. Evidence from our laboratory demonstrates that in a number of routinely used cell lines phosphodiester oligonucleotides are degraded within minutes by nucleases found in the plasma membrane, cytoplasm and in nuclei (Hoke *et al.*, in press).

Second, a variety of chemically modified oligonucleotides have been reported to be active in cell culture. Although considerable variation has been reported, phosphorothioate oligonucleotides appear to be more potent than methylphosphonate oligonucleotides. Conjugation of alkylators and interchelators to phosphodiesters and methylphosphonates has been reported to increase potency. Many of these modifications have been positioned at either the 3′ or 5′ end of the oligonucleotides; 3′ positioning is an attempt to increase stability to 3′-exonuclease, the predominant serum nuclease. Lipophilic and poly(L-lysine) conjugates have also displayed enhanced potencies presumably via some modulation of cellular pharmacokinetic characteristics.

Third, oligonucleotides have demonstrated activities against a broad array of viral targets, oncogenes, normal cellular gene products and various transfected genes. This array of pharmacological effects clearly demonstrates the broad potential therapeutic applicability of these drugs.

Fourth, although the data from studies included in Table I are limited, when it is combined with *in vitro* toxicologic data (Crooke, 1991), the therapeutic indexes of phosphorothioate oligonucleotides appear to be quite high. Initial data regarding certain phosphorothioates of 20 and 21 nucleosides in length, targeted to human papilloma virus and herpes simplex virus, respectively, also demonstrate that these compounds are extremely well tolerated in animals (Mirabelli *et al.*, in preparation). The effects of specific base composition within an oligonucleotide, oligonucleotide length, specific chemical modifications in oligonucleotide and cellular parameters (i.e. cell type, cell cycle phase and stages of differentiation) on the potential toxicology and non-antisense activities of these compounds are not yet clearly defined (Crooke, 1991).

Fifth, very little data that support putative mechanisms of action have been reported and generalizations concerning precise mechanisms of action are not possible. A variety of mechanisms have been proposed to explain the ultimate pharmacologic action of antisense oligonucleotides, all resulting from the hybridization of the drug with the complementary sequence within a target RNA. These mechanisms include the disruption of ribosomal assembly and function, formation of an RNase H substrate and subsequent cleavage of the target RNA, and disruption of RNA splicing processes or other RNA metabolic processes. It is very likely that many 'terminating' mechanisms can be exploited for the cellular action of antisense oligonucleotides and that the mechanisms of a particular oligonucleotide are the result of the particular RNA and sequence target, the cell in which the drug is acting and the chemical structure of the oligonucleotides.

Examples of antisense pharmacologic activities

Our laboratory has demonstrated activities of oligonucleotide drugs against a number of molecular disease targets. Below is a brief summary of work on two targets: herpes simplex virus and a human cell adhesion molecule, ICAM-1. These data are reviewed in an attempt to provide examples of the antisense drug discovery process and the activities of antisense compounds directed against viral gene targets and host gene targets.

Antisense oligonucleotides directed to herpes simplex virus RNA targets

In vitro activities. Smith & Smith (1986) first reported antisense inhibition of HSV replication using oligonucleotides targeted to the splice junction sequences of the HSV-1 1E4 and 1E5 pre-RNAs. It was later reported that increasing the length of the oligonucleotide increased the antiviral activity against HSV-1 (Kulka *et al.*, 1989). The most active oligonucleotide, a 12-nucleotide long oligomethylphosphonate, was directed against a splice junction covering six nucleotides in both exon and intron. The potency of the compound was greatest when added at the time of infection ($IC_{50} = 15\ \mu M$) with a 5- to 10-fold reduction in potency when the oligonucleotide was added 1 h post-infection. A 20% inhibition in splicing was observed in oligonucleotide treated infected cells *versus* untreated infected cells. Conjugation of the 12-mer oligomethylphosphonate with a psoralen-derivative increased the potency of the compound approximately 3-fold relative to the unconjugated compound. However, the psoralen conjugate required activation by UV irradiation following addition to the infected cells.

A study by Draper *et al.* (1990) using phosphodiester oligonucleotides complementary to two related region of the HSV-1 Vmw 65 mRNA, reported that an oligonucleotide targeted to the translation initiation region effectively inhibited HSV-1 replication. The other oligonucleotide was inactive, causing these authors to conclude that sequences within the same mRNA can exhibit differential sensitivities to antisense oligonucleotides.

Our laboratory has designed and tested several oligonucleotides which are complementary to the translation initiation regions of several mRNAs of HSV. Oligonucleotides which target the HSV UL13 mRNA were found to be effective inhibitors of HSV replication, as measured in an infectious yield assay (Draper & Brown-Driver, 1991; Draper *et al.*, submitted). The protein encoded by the UL13 gene has been putatively identified as a phosphotransferase which may be involved in the phosphorylation of viral capsid proteins (Smith *et al.*, 1986; Stevely *et al.*, 1985). Preliminary screening experiments revealed that phosphorothioate oligonucleotides were significantly more potent than phosphodiester and methylphosphonate oligonucleotides (Draper & Brown-Driver, 1991; Draper *et al.*, submitted). One of the most potent compounds evaluated was ISIS 1082, a 21-mer phosphorothioate oligonucleotide, targeted to a secondary initiation codon present in HSV-1 and HSV-2 UL13 mRNA. This compound inhibited both HSV-1 (KOS strain) and HSV-2 (HG52 strain) replication in an infectious yield assay. Site specific cleavage of synthetic UL13 transcripts was induced by addition of ISIS 1082 in RNA processing extracts of HeLa cells suggesting that ISIS 1082 may inhibit expression of the UL13 gene product by inducing RNAase H specific cleavage of UL13 mRNA.

Evaluation of the compound in infectious yield assays using acyclovir sensitive and resistant strains and in comparative dose responses with acyclovir and other phos-

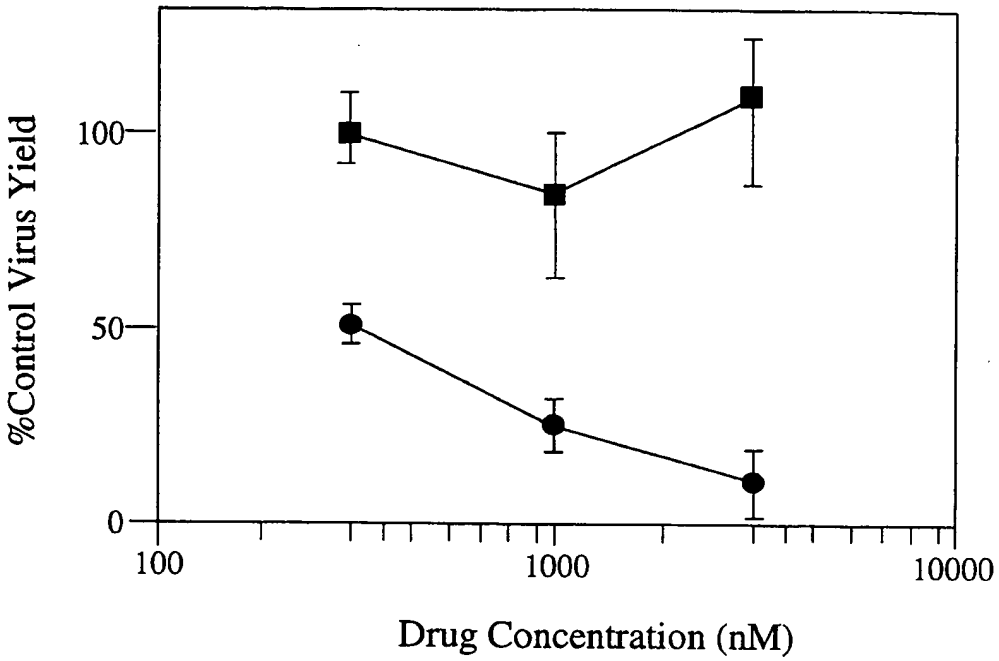

Figure 1 Sensitivity of an acyclovir resistant strain of HSV-1 (DM 2.1, thymidine kinase deletion mutant) to ISIS 1082 (●) and acyclovir (■). Activities were measured in an infectious yield assay and expressed as a percent of untreated infected cell virus yield

phorothioate oligonucleotides provided more evidence that ISIS 1082 produces its antiviral activity via a sequence specific antisense effect. First, ISIS 1082 inhibits the replication of the HSV-1 strain KOS in HeLa cells by 50% and 90% at concentrations of approximately 300 nM and 2 μM, respectively. ISIS 1082 was 3- to 10-fold more potent then a phosphorothioate oligonucleotide of similar length and equivalent (but scrambled) nucleotide base composition when tested against certain strains of HSV-1 and HSV-2. In addition, it was found that ISIS 1082 was active against a number of acyclovir resistant strains of HSV-1. Figure 1 shows the activity of ISIS 1082 against the thymidine kinase deletion mutant strain, DM2.1. Acyclovir did not inhibit the replication of this strain. However, treatment with ISIS 1082 resulted in a dose-dependent decrease in infectious viral yield that was equivalent to that observed against the KOS strain of HSV-1. At concentrations as high as 100 μM of ISIS 1082, only minimal effects on host cell growth and metabolism were observed (Crooke *et al.*). This lack of *in vitro* toxicity is again consistent with the postulated highly selective mode of action of the antisense compound.

In vivo activities. Earlier preliminary reports have suggested *in vivo* activities of antisense drugs against HSV infections. One report indicated that an oligomethylphosphonate was active in a mouse model of herpes simplex virus 1 infection (Kulka *et al.*, 1989). Two additional laboratories have reported on the activity of phosphorothioates against HSV-1 infections in mouse models of ocular herpetic keratitis (Kimura *et al.*, 1991; Metcalf *et al.*, 1991).

Recent data have demonstrated that topical application of ISIS 1082 in an aqueous buffer to the cornea of mice infected with HSV-1 (KOS) resulted in curative activity at drug concentrations of 0.3% and 5% (Brandt *et al.*, 1991; Brandt *et al.* submitted). The activity of ISIS 1082 in this model was equivalent to trifluorothymidine and exhibited no local or systemic toxicities. ISIS 1082 is currently being studied in rabbit models of HSV-1 induced epithelial keratitis and other animal models of dermal and systemic HSV infection to better define the pharmacology of the compound.

Antisense oligonucleotides directed to intercellular adhesion molecules

To date most reports of antisense oligonucleotide activities in non-viral infection models have focused on oncogene targets and receptor signaling targets as seen in Table I. Our laboratory has recently explored the use of antisense oligonucleotides to pharmacologically manipulate the expression of certain cellular adhesion molecules (Chiang *et al.*, 1991).

Rationale for adhesion molecules as antisense targets

The binding of circulating leukocytes to vascular endothelium is an obligatory step in the emigration of leukocytes out of the vasculature to the site of infection or injury (Harlan, 1985). Several endothelial proteins have recently been identified which mediate the adherence of leukocytes to inflamed vascular endothelium and subsequent migration out of the vasculature (Stoolman, 1989; Osborn, 1990; Springer, 1990). One such protein, ICAM-1, is a 95–105 kD glycoprotein first identified by the ability of a monoclonal antibody to block phorbol ester induced aggregation of a B-cell line (Rothlein *et al.*, 1988). The cellular distribution of ICAM-1 is different from other endothelial cell adhesion molecules in that it is expressed in both endothelial cell and non-endothelial cells including leukocytes, fibroblasts, kernatinocytes and other epithelial cells (Table II). ICAM-1 binds circulating leukocytes through LFA-1 (CD11a, CD18), a member of the β_2 integrin family (Marlin & Springer, 1987). ICAM-1 is a member of the immunoglobin gene superfamily containing five immunoglobulin domains (Simmons *et al.*, 1988; Staunton *et al.*, 1988; Tomassini *et al.*, 1989). Expression of ICAM-1 is inducible by a number of cytokines including IL-1, TNF-α and IFN-γ (Rothlein *et al.*, 1988; Stoolman, 1989; Osborn, 1990; Springer, 1990). The broad tissue distribution of ICAM-1 suggests that it is not only involved in the emigration of leukocytes out of the vasculature, but may play a more extensive role in immune responses. Additional roles suggested for ICAM-1 include localization of leukocytes to the area of inflammation in extravascular spaces, enhancement of the recognition of antigen presenting cells by T lymphocytes, formation of lymphocyte germinal centers, enhancement of natural killer cell response and differentiation of thymocytes (Rothlein *et al.*, 1986; Dustin *et al.*, 1986 & 1988; Makgoba *et al.*, 1988; Altmann *et al.*, 1989; Boyd, 1989; Robertson *et al.*, 1990; Springer, 1990). In addition ICAM-1 is the receptor for over 90% of the rhinovirus serotypes (Staunton *et al.*, 1989; Tomassini *et al.*, 1989).

***In vitro* inhibition of ICAM-1 expression by antisense oligonucleotides**

During the initial evaluation of a series of phosphorothioate oligonucleotides targeted to specific sites within the ICAM-1 mRNA it was found that the cationic lipid,

Table II Leukocyte adhesion molecules

Endothelial CAM	*Expressed on other cells*	*Gene family*	*Induction kinetics*	*Leukocyte ligand*	*Type of leukocyte bound*
ICAM-1	Keratinocytes, fibroblasts, leukocytes, etc.	Immunoglobulin	4 h to 72 h	LFA-1, MAC-1	Lymphocytes, monocytes, granulocytes
ICAM-2	Activated lymphocytes	Immunoglobulin	Constitutively	LFA-1	Lymphocytes, monocytes, granulocytes
VCAM-1	No	Immunoglobulin	4 h to 72 h	VLA4	Lymphocytes, monocytes
ELAM-1	No	LEC-CAM	2 h to 18 h	Carbohydrate	Granulocytes monocytes, memory T cells
GMP-140	Platelets	LEC-CAM	5 min to 2 h	Carbohydrate	Granulocytes, monocytes

DOTMA markedly enhanced the activity of the antisense oligonucleotides used in this study. DOTMA was originally described as a vehicle for transfection of DNA into cells (Felgner *et al.*, 1987). Cationic lipid delivery methods differ from normal liposomal delivery methods, in that the DNA or oligonucleotide is not encapsulated within the liposome, but rather is associated with the surface of the liposome through ionic interactions. Preliminary data in certain cell lines indicate that DOTMA enhances cell association of oligonucleotides at least 10-fold and markedly changes the intracellular distribution of the oligonucleotide, with apparently less oligonucleotide being concentrated in endosomes or lysosomes and more found in the nucleus (Chiang *et al.*, 1991; Bennett *et al.*, in preparation). Therefore, in some cells DOTMA will enhance oligonucleotide entry into the cytoplasm of cells similar to direct microinjection. The use of DOTMA has the advantage over microinjection experiments in that oligonucleotides can be introduced into large number of cells allowing biochemical analysis to be performed. In addition, it was determined that DOTMA had no effect on the expression of ICAM-1 when used at concentrations that maximized oligonucleotide uptake and activity (Chiang *et al.*, 1991). The use of DOTMA in these experiments allowed us to determine which regions on the ICAM-1 mRNA serve as the best target sites for antisense oligonucleotides and determined the mechanism by which antisense oligonucleotides inhibit ICAM-1 expression. To our knowledge this is the first report demonstrating that cationic lipids enhance antisense oligonucleotide activity in mammalian cells.

Using DOTMA as a formulation medium we have demonstrated that antisense oligonucleotides which target human ICAM-1 mRNA inhibit the expression of ICAM-1 in two cell culture systems HUVEC and a human lung carcinoma, A549 (Chiang *et al.*, 1991). Screening antisense oligonucleotides which target a number of sites on the ICAM-1 mRNA revealed that two sites were especially sensitive to inhibition with antisense oligonucleotides; the AUG translation initiation codon and specific sequences in the 3′-untranslated region. Data from these studies suggest that hybridization affinity is important for antisense oligonucleotides, as truncated versions of active oligonucleotides (<20-mers) exhibit decreased activity, however, hybridization affinity is not sufficient to ensure antisense activity. Therefore, target site selection is also an important parameter to consider when designing antisense oligonucleotides.

The most active ICAM-1 antisense oligonucleotide targets the 3′-untranslated region of the ICAM-1 mRNA. ISIS 1939 hybridizes to the ICAM-1 mRNA, nearly 300 bases 3′- to the translation termination site, therefore it should not directly affect translation of the protein. This oligonucleotide was shown to inhibit the expression of ICAM-1 in endothelial cells as measured by ELISA using a monoclonal antibody to ICAM-1 (Figure 2). Under equivalent experimental conditions treatment of endothelial cells with ISIS 1939 blocked the adhesion of HL60 cells. Thus the blockade of ICAM-1 expression was coincident with the loss of functional activity of the protein. Oligonucleotides which hybridized to other sequences in the 3′-untranslated region of ICAM-1 mRNA were not as effective as ISIS 1939 (Figure 2). Therefore, the effects of ISIS 1939 are unique to the target site to which it hybridizes.

ICAM-1 mRNA contains three repeats of a consensus sequence, AUUUA, thought to be involved in destabilization of mRNA (Caput *et al.*, 1986; Shaw & Kamen, 1986; Brawerman, 1989). An oligonucleotide that targets those sequences was shown to exhibit weak activity. Howevef, ISIS 1939 targets an area approximately 200 bases 5′- to the AUUUA sequences. The region targeted by ISIS 1939 is predicted to be a stable stem loop structure which when bound would disrupt the structure. Analysis of steady state mRNA levels from oligonucleotide treated cells revealed that ISIS 1939

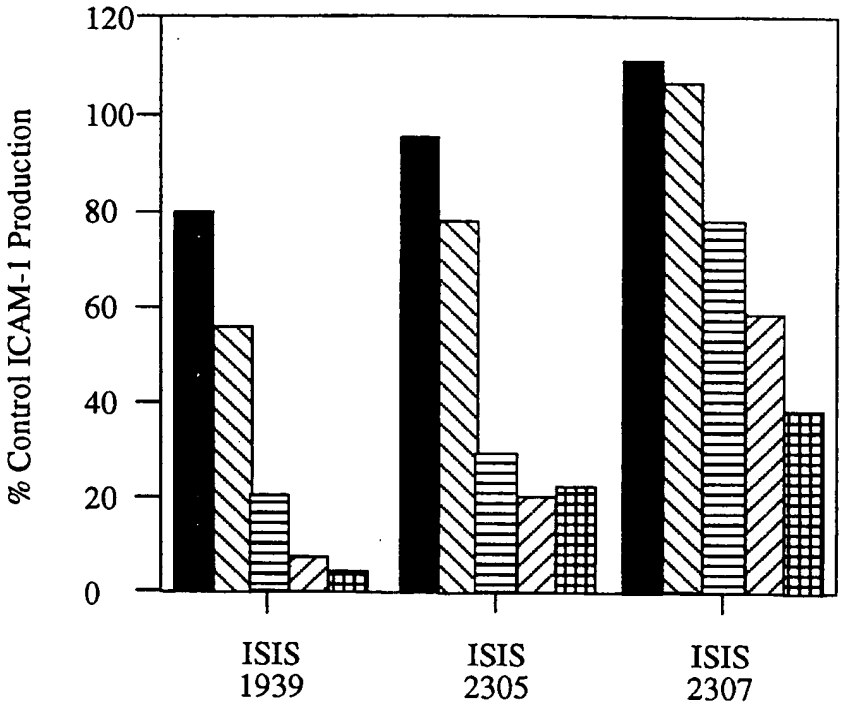

Figure 2 Inhibition of IL-1 induced ICAM-1 expression in A549 cells with antisense oligonucleotides which hybridize to the 3'-untranslated region of ICAM-1 mRNA. Cells were treated with phosphorothioate oligonucleotides (20 mers) at concentrations of ■ 0.1 μM ▨ 0.3 μM ▤ 0.5 μM ▨ 0.7 μM ▦ 1.0 μM in the presence of DOTMA. ICAM-1 expression was measured by ELISA using an ICAM-1 monoclonal antibody 84H10

specifically reduced the quantity of ICAM-1 mRNA per cell. The reduction of ICAM-1 mRNA was not due to decreased transcription of the ICAM-1 gene as analysed by nuclear run-off reactions. Therefore, ISIS 1939 must destabilize the ICAM-1 mRNA either by an RNase H dependent mechanism and/or by modulating natural processes which help to stabilize the ICAM-1 mRNA.

Oligonucleotides targeted to certain other specific sites within ICAM-1 mRNA were found to be potent inhibitors of ICAM-1 protein expression and cell adhesion. These oligonucleotides were targeted to sequences within the 5' untranslated region and the translation initiation region. The oligonucleotide targeted to the translation initiation region did not cause a reduction in the steady state level of ICAM-1 mRNA; unlike that found with ISIS 1939. Taken together these data suggest that different oligonucleotides targeted to different sites on an RNA may inhibit the production of a protein by different mechanisms.

Summary

The notion of using antisense oligonucleotides as pharmacologic agents is a derivative of the central dogma of molecular biology and knowledge of the physical and

chemical properties that govern the structure of nucleic acids. The practical evidence that antisense oligonucleotides can be drugs is a result of the work of a number of laboratories, including those cited in this review.

Key to the continued progress in the field of antisense therapeutics is the realization that oligonucleotides and their RNA targets work via the same principles of pharmacology that govern the actions of all other classes of drugs. Considering the properties of drugs that define their pharmacologic value, such as ligand-receptor binding affinity and fidelity and realizing the intrinsic properties of oligonucleotides, it is very clear that these compounds have enormous potential value in treating human diseases.

During the next few years a number of oligonucleotide compounds will enter into clinical trials. These first generation antisense drugs (e.g. phosphorothioates) will encounter many of the same issues and hurdles that confront all novel pharmaceutical agents; large-scale process development, adequate methods and tools to define clinical pharmacokinetics and metabolism, etc. Another important component of this process is the continued examination and definition of the molecular pharmacodynamics and pharmacokinetics of these drugs. We need to better understand how the structure and function of RNA defines the sensitivity of specific target sites to antisense oligonucleotides, the precise role of RNase H and other intracellular enzymes and proteins in the mechanism of action in oligonucleotides, the process by which oligonucleotides penetrate cellular membranes and distribute in cells, the non-sequence specific interactions that oligonucleotides can engage in both in and out of cells, and the metabolic pathways (both nuclease and non-nuclease) and metabolites that are likely to play a role in the metabolism of antisense drugs. The combination of this molecular, cellular, and clinical information will allow us to better determine the specific molecular targets and diseases that can be successfully treated with the first generation of antisense drugs. As important, it will define the biology, chemistry, and pharmacology of second and third generation antisense drugs.

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THERAPEUTIC APPLICATIONS OF OLIGONUCLEOTIDES

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INTRODUCTION

First proposed in 1978 by Zamencik & Stephenson (1), oligonucleotide therapeutics represent a new paradigm for drug discovery. The technology focuses on a class of chemicals, oligonucleotides, that has not been studied as potential drugs before and employs them to intervene in biological processes that likewise have not been studied previously as sites at which drugs might act.

The paradigm has resulted in substantial enthusiasm because oligonucleotides may display dramatic increases in affinity and selectivity for their nucleic acid targets compared to traditional drugs. Furthermore, antisense technology may facilitate rational drug design. Table 1 compares affinities and the potential for selectivity of oligonucleotides versus traditional drugs. The comparison is based on average affinities of typical traditional drugs in optimized assays with purified receptors and data derived from a 21-mer phosphorothioate oligonucleotide in binding assays performed in 1M NaCl. Hybridization varies substantially as a function of ionic strength, and the affinities at 100 mm NaCl in the presence of Mg^{2+} for the 21-mer are significantly lower. Furthermore, affinities may be lower in physiological systems with RNA that has secondary structure, so these comparisons present the opportunity in its broadest dimensions.

A number of terms have been coined and often misused to describe various

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Table 1 Affinity and selectivity of traditional and oligonucleotide drugs. Affinity constants were determined as described in text

Traditional drug		Oligonucleotide drug (1M NaCl)	
Affinity for receptor	10^8	Affinity for receptor sequence	10^{29}
Affinity for isotype	10^6 –10^8	Affinity for one base mismatch	10^{20}
Maximum affinity for other proteins	10^6 –10^8	Maximum affinity for nucleic acid binding proteins	10^{12}–10^{13}
ΔKd	1–10^2	Δ kD	10^6–10^{11}

components of the overall approach to using oligonucleotides as therapeutic agents. *Antisense* describes the interaction between oligonucleotides complementary to (sense) pre-mRNA[1] or mRNA molecules; these inhibit the oligonucleotides production of the protein product. The term has been broadened to describe any therapeutic oligonucleotide interaction with nucleic acids. *Triplex* denotes the interaction between oligonucleotides and double-stranded DNA that may result in inhibition of transcription. RNA structures display double-stranded regions, however, and thus the formation of triple-stranded structures in RNA is also possible. *Aptamers* describes the use of oligonucleotides to bind to nucleic acid binding proteins.

SCOPE AND OBJECTIVES OF THE REVIEW

This review focuses strictly on the use of oligonucleotides designed to interact with nucleic acids as therapeutics. The objectives are to place oligonucleotide therapeutics in the context of modern drug discovery and development and to summarize recent progress.

[1]Abbreviations: BFGF, basic fibroblast growth factor; cAMP, cyclic AMP; CH_3-P, methylphosphonate oligonucleotides; EGF, epidermal growth factor; ELAM, endothelial cell adhesion molecule; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; HB, hepatitis B; HIV, human immunodeficiency virus; HSV, herpes simplex virus; HTLV, human T-cell lymphotrophic virus; ICAM, intracellular adhesion molecule; IGF, insulin-like growth factor; IL-1, interleukin 1; introns, intervening sequences; IV, Influenza virus; mRNA, messenger ribonucleic acid; P, phosphodiester oligonucleotides; P-acridine, phosphodiester oligonucleotide conjugated with acridine moiety; PCNA, proliferating cell nuclear antigen; PLA_2, phospholipase A_2; P-lipid, phosphodiester oligonucleotide conjugated with lipid moiety; PMA, phorbol mysteric acid; P-S, phosphorothioate oligonucleotides; Rev, regulation of virion protein expression; RSV, Rous sarcoma virus; TAR, Tat response element; TBE, tick-borne encephalitis; Tm, thermal transition point; VSV, vesicular stomatitis.

BASIC CONSIDERATIONS

Conceptually, oligonucleotide drug effects can be rationalized by traditional receptor theory and basic concepts concerning drug action. Within the broad context of pharmacological theory, however, a number of differences influence rational drug design and the potential utility of these agents.

Pharmacodynamics

RNA INTERMEDIARY METABOLISM Oligonucleotides are designed to modulate the information transfer from the gene to protein—in essence, to alter the intermediary metabolism of RNA. Figure 1 summarizes these processes.

RNA intermediary metabolism is initiated with transcription. The transcription initiation complex contains proteins that recognize specific DNA sequences and locally denature double-stranded DNA, thus allowing a member of the RNA polymerase family to transcribe one strand of the DNA (the antisense strand) into a sense pre-mRNA molecule. Usually during transcription, the 5' end of the pre-mRNA is capped by adding a methyl-guanosine and most often by methylation of one or two adjacent sugar residues. This enhances the stability of the pre-mRNA and may play a role in a number of key RNA processing events (2). Between the 5' cap and the site at which translation is initiated is usually a stretch of nucleotides; this area may play a key role in regulating mRNA half-life (3).

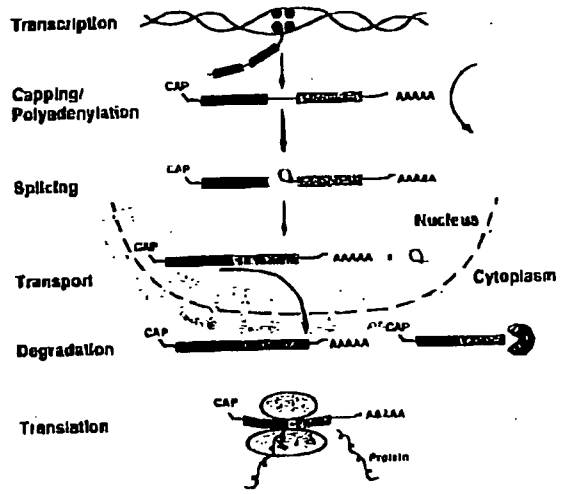


Figure 1 RNA processing.

Similarly, the 3' end of the pre-mRNA usually has a stretch of several hundred nucleotides beyond the translation termination signal. This area often plays an important role in determining mRNA half-life. Moreover, post transcriptionally, most pre-mRNA species are polyadenylated. Polyadenylation stabilizes the RNA, is important in transport of mature mRNA out of the nucleus, and may play important roles in the cytoplasm as well (4, 5).

Because eukaryotic genes usually contain intervening sequences (introns), most pre-mRNA species must have these sequences excised and the mature RNA spliced together. Splicing reactions are complex, highly regulated, and involve specific sequences, small molecular weight RNA species, and numerous proteins. Alternative splicing processes are often used to produce different mature mRNAs and, thus, different proteins. Even though introns are often considered waste, important sequences are conserved, and some introns may play a variety of regulatory roles.

Mature mRNA is exported to the cytoplasm and engages in translation. mRNA half-life varies from a few minutes to many hours and appears to be highly regulated (3).

Each step shown in the pathway is a composite of numerous steps, and each step is theoretically amenable to intervention with oligonucleotides. For virtually no mRNA is the pathway fully defined, however, and available information is insufficient to determine the rate-limiting steps in the intermediary metabolism of any mRNA species (6, 7).

AFFINITY The affinity of oligonucleotides for their receptor sequences results from hybridization interactions. The two major contributors to the free energy of binding are hydrogen bonding (usually Watson-Crick base pairing) and base stacking in the double helix that is formed. As mentioned, affinity is affected by ionic strength. Affinity results from hydrogen bonding between complementary base pairs; the reduction in entropy results from the stacking of the coplanar bases. Consequently, affinity increases as the length of the oligonucleotide receptor complex increases. Affinity also varies as a function of the sequence in the duplex. Nearest neighbor rules allow the prediction of the free energy of binding for DNA-DNA and RNA-RNA hybrids with relatively high precision (8, 9). Less information is available to develop predictions for DNA-RNA duplexes. A common misconception is that DNA-RNA duplexes are more stable than DNA-DNA duplexes. In fact, the relative stability of these duplexes varies as a function of the sequence. RNA-RNA duplexes are typically the most stable (S. M. Freier, unpublished results).

As with other drug-receptor interactions, activity requires a minimum level of affinity. For many targets and types of oligonucleotides, the minimum length of an oligonucleotide may be 12–14 nucleotides.

SPECIFICITY Specificity derives from the selectivity of Watson-Crick or other types of base pairing. The decrease in affinity associated with a mismatched base pair varies as a function of the specific mismatch, the position of the mismatch in a region of complementarity, and the sequence surrounding the mismatch. As an example, Table 2 compares the impact of various mismatches centered in two complementary 18-mers. The $\Delta\Delta G^0_{37}$ or change in Gibbs free energy of binding induced by a single mismatch varies from $^+0.2$ to $^+4.9$ kcal/mol per-modification at 100 mM NaCl. Thus, a single base mismatch results in a change in affinity of approximately 500-fold (10). Modifications of oligonucleotides may alter specificity. In fact, we have synthesized modified bases with substantially enhanced selectivity and others that display virtually no preferences for different bases.

Table 2 Effects of single-base mismatches on duplex stability. Absorbance vs temperature profiles were measured at 4 μM each strand in 100 mM Na^+, 10 mM phosphate, 0.1 mM, EDTA, pH 7.0.

X strand: 5'-d(CTC GTA CCx TTC CGG TCC)-3' fully phosphorothioate X = dA, dC, dG, or T

Y strand: 5'-(GGA CCG GAA yGG TAC GAG)-3' fully RNA y = A, C, G, or U

X	Y	T_m[1] (°C)	ΔT_m (°C)	ΔG°_{37} (kcal/mol)	$\Delta\Delta G^\circ_{37}$ (kcal/mol)
dT	rA	53.6	—	−11.6	—
dT	rC	40.8	−12.8	−8.9	+2.6
dT	rG	50.0	−3.6	−10.5	+1.1
dT	rU	41.9	−11.7	−9.1	+2.5
dG	rC	56.9	—	−13.1	—
dG	rA	42.3	−14.6	−8.9	+4.2
dG	rG	45.0	−11.8	−9.3	+3.8
dG	rU	45.7	−11.1	−9.8	+3.3
dC	rG	59.0	—	−12.5	—
dC	rA	43.3	−15.7	−9.3	+3.2
dC	rC	39.5	−19.4	−8.7	+3.9
dC	rU	40.0	−19.0	−8.7	+3.8
dA	rU	52.7	—	−11.4	—
dA	rA	42.7	−10.0	−9.3	+2.2
dA	rC	42.7	−10.0	−9.1	+2.3
dA	rG	44.5	−8.1	−9.3	+2.1

[1] T_ms and free energies of duplex formation were obtained from fits of the absorbance vs. temperature data to a two-state model with linear sloping baselines. Reported parameters are averages of at least three experiments.

Based on the differences in affinity of oligonucleotides for their complementary target sequence, calculations suggest that unmodified oligodeoxynucleotides between 11–15 in length should be able to bind selectively to a single RNA species in the cell (11). Studies in our laboratories have demonstrated that affinities predicted by nearest neighbor analyses are highly useful in rational drug design (10). For example, by using strategies based on nearest neighbor predictions, oligonucleotides can be designed that selectively inhibit the production of mutant RAS containing a single base change in the mRNA vs. normal RAS in cells in tissue culture (B. P. Monia et al, manuscript submitted).

NUCLEIC ACID SELECTIVITY The 2'-hydroxyl in RNA results in the sugar assuming a different conformation from that in DNA. RNA-RNA duplexes assume an A-form double helix whereas DNA-DNA duplexes assume a B-form double helix. Consequently, oligonucleotides can be modified to bind more tightly to RNA or DNA sequences. For example, Table 2 shows the effect of 2'-modifications at every position of a 15-mer on T_m and ΔG°_{37} for DNA and RNA targets and demonstrates that 2'-O-methyl substitutions increase T_m for RNA by 1.4° per modification compared to DNA (12).

RNA STRUCTURE RNA can assume a variety of secondary structures deriving from intramolecular base pairing. The simplest structures are stem-loops in which double-stranded regions are interspersed with loops and random coils. More complex structures described as *pseudoknots* also form (13). These structures are profoundly important in determining RNA function and influencing the ability of oligonucleotides to bind to their RNA targets. The types of effects of bound oligonucleotides on RNA function are affected by RNA structures as well.

Pharmacokinetics

As with any other class of drugs, oligonucleotide drugs must attain a sufficient concentration at their receptor for a sufficient period to display activity. Inasmuch as most of the targets for oligonucleotides are intracellular, oligonucleotides must be relatively stable in and outside the cell and must be able to traverse the cellular membrane.

NUCLEASE STABILITY Oligonucleotides may be degraded by nucleases. Nucleases that degrade DNA or RNA from either the 5' or 3' terminus are known as *exonucleases;* those that cleave internally are *endonucleases.* Numerous nucleases exist and have been shown to degrade oligonucleotides. Although in serum the dominant nuclease activity is 3' exonuclease, in cells and other bodily fluids 3' and 5' exonucleases and endonucleases are present.

In serum, phosphodiester oligodeoxynucleotides are rapidly degraded. The rate of degradation varies as function of the sequence and length of the oligonucleotide and the type of serum (14–16). Typically, half-lives of phosphodiester oligodeoxynucleotides range from 15 to 60 min in most sera. Heat inactivation of serum reduces the rate of degradation of oligonucleotides. Oligoribonucleotides are significantly less stable than oligodeoxynucleotides.

Work from many laboratories has demonstrated that a wide range of modifications may be used to enhance the stability of oligonucleotides. Phosphate modifications have been shown to result in marked increases in stability (see Table 3). Phosphorothioate oligonucleotides have been shown to be extremely stable in media, cells and cell extracts, serum, various tissues, urine and stable to most nucleases (16–20). The half-life of phosphorothioate oligonucleotides is greater than 24 hr in nearly all environments tested. Furthermore, phosphorothioates have been shown to be stable to various restriction endonucleases when in duplexes. In general, one of the diastereomers is cleaved slowly and the other is entirely resistant (21–24).

The non-ionic methylphosphonate analogs have also been shown to be extremely stable to nucleases (25–31). Again, these oligonucleotides are diasteresomeric at each modified phosphate, and the R isomer is slightly more sensitive than the S isomer to degradation by nucleases (16, 32).

Table 3 Effects of 2' modifications on hybridization and stability. Duplex hybridization was evaluated from absorbance vs. temperature profiles at 260 nm in 100 mM Na^+, 10 mM phosphate, 0.1 mM EDTA, pH 7.0 at 8 μM strand concentration.

		Hybridization		Serum stability
Modification[1]	Positions	T_m[2] vs DNA (°C)	T_m vs RNA (°C)	$T_{1/2}$
Phosphodiester	All	50.5	39.0	1 h
Phosphorothioate	All	43.2		>24 h
2'-O-nonyl dA	12 13 14		41.3	60 h
2'-O-allyl	12 13 14	50.3	40.8	10 h
2'-O-benzyl	12 13 14	45.5	37.8	18 h
2'-O-aminopropyl	12 13 14	53.7	42.0	
				1 h
2'-fluoro P + S	2'F in 12 13 14 P = S in all	47.2	36.5	>>24 h

[1] 15 mer: CGA CTA TGC AAA AAC

[2] T_m is the temperature at which half the strands are in the duplex state and half are in the coil state. T_m was obtained from a nonlinear least squares fit of the experimental data to a modified two-state model with linear sloping baselines (224)

Other classes of modifications that have been reported to result in substantial nuclease stability include the phosphoramidates (19, 33) and isopropyl phosphate triesters (34, 35). Interestingly, ethylphosphate triesters were shown to be cleaved after being deethylated in cells (27, 36). Oligonucleotides containing α-anomers in the sugar moiety are substantially more stable in serum and cells than natural phosphodiesters (14–15, 37–41).

Modifications at the 2'-position of the sugar have also been shown to enhance nuclease stability (42). 2'-O-methyl-oligonucleotides were shown to be significantly more resistant than unmodified oligonucleotides, and 2'-O-allyl modified oligonucleotides were even more stable (43). In studies in our laboratory, a large number of 2'-modifications have been characterized. 2'-O-methyl analogs were highly resistant to nucleases in serum and cells. Modifications as bulky as nonyl groups were shown to have only a minor negative effect on hybridization and to impart high levels of nuclease stability In contrast, 2'-fluoro derivatives were nearly as sensitive to nucleases as unmodified oligoribonucleotides (12, 44). Table 3 provides a comparison of affinities to RNA and stabilities in serum for several 2' modified oligonucleotides (45). Although numerous other modifications have been studied, either insufficient data concerning hybridization properties or nuclease stabilities have been reported to support conclusions or their hybridization properties were unattractive. For example, open ring sugar analogs of adenosine were reported to be nuclease stable (46). Acyclic pentofuranosyl modified oligonucleotides were reported to be nuclease resistant, but the T_m for these oligonucleotides was reduced 9–15 degrees per modification (47). Other acyclic sugars have been reported but, again, the hybridization properties were poor (48). Carbocyclic modified oligothymidylates were reported to be nuclease resistant and to hybridize to oligodeoxyadenosine with higher affinity than natural oligodeoxy-thymidylate (49, 50), but studies on mixed sequences have not been reported.

A wide variety of phosphate replacements have also been studied. In earlier work, the phosphodiester was replaced with esters, amides, and various polymeric materials, but these modifications were not designed to be used as antisense oligonucleotides and, therefore, are largely unattractive (12, 16). More recently, formacetal replacement of the phosphate has been reported by two groups to result in oligonucleotides with acceptable hybridization properties and nuclease resistance (51–53).

Other modifications for which little information is available include sulfonamide replacement of phosphate (54), diphosphonate dinucleotides (55), acetamide linkages (56, 57), and phosphonyl methyl linkages (58). These and other modifications are discussed in detail in two recent reviews (12, 16). In our laboratories, a number of other novel backbone modifications have been synthesized. Given the number of novel synthetic approaches and molecules

and the number of laboratories now involved, a substantial increase in the repertoire of backbone-modified oligonucleotides with desirable properties is likely in the near future.

In addition to uniform modifications, a number of pendant groups at the 5' and/or 3' termini and more recently in internal positions of oligonucleotides have been reported to enhance nuclease stability. Modifications include intercalating agents (59–62) and poly-L-lysine (63, 64) at the 5' or 3' terminus and a number of modifications such as amino-alkoxy (65), anthraquinone (66), and alkyl groups (45). Moreover, heterocycle modifications, including pendant groups from the N2 site of guanine (67, 68), pendant groups from 3-deazaguanine (69), and 5- and 6-position modifications of deoxycytidine and thymidine (70), have shown increased stability to nucleases of varying levels.

In conclusion, numerous medicinal chemical strategies can be employed to create oligonucleotides with varying degrees of nuclease stability. The choice of the modification(s) employed is dictated by the level of stability desired and other desired properties of the oligonucleotides. It is now possible to design oligonucleotides that display excellent hybridization characteristics and half-lives, that range from minutes to several days when oligonucleotides are incubated with nucleases, serum, cells, or cell extracts.

INTRACELLULAR STABILITY Although considerable confusion and controversy exist with regard to the stability of oligonucleotides in cells in tissue culture and the ability to predict intracellular stability of oligonucleotides based on stability in sera, a consensus opinion is emerging. The nuclease activity of sera derived from different species varies. Fetal calf serum is more active than mouse serum, and human serum appears to have the least nuclease activity (G. D. Hoke, unpublished observations). All sera display substantial nuclease activity, however, and there are significant lot-to-lot variations. In all sera tested, 3' exonucleases constitute the primary nuclease activity (12, 16, 71). In a number of publications, fetal calf serum used in tissue culture experiments has been heated to inactivate nucleases. Again, however, conditions were not standardized, and in some lots of sera, heating to 65°C for 30 min does not inactivate all nucleases (16).

Another factor that has contributed to confusion is that a variety of labeling methods and analytical techniques have been employed. Studies have employed $3'^{32}P$ and $5'^{32}P$ labeled oligonucleotides, $5'^{35}S$ labeled oligonucleotides, and oligonucleotides labeled with fluorescent pendant groups at the 5' terminus (14–16, 72). Relatively few studies have used uniformly labeled oligonucleotides. Furthermore, relatively few studies have rigorously separated intact oligonucleotides from degradation products, and even fewer have performed careful kinetic studies.

Studies in our laboratory have employed either phosphodiester oligonucleotides uniformly labeled with ^{32}P or phosphorothioate oligonucleotides uniformly labeled with ^{35}S. The kinetics of degradation have been studied with several cell lines in vitro and cytoplasmic and nuclear extracts derived from HeLa cells. In contrast to a number of studies, in all cells studied to date, phosphodiester oligonucleotides were degraded within 15–30 min of incubation (71, 73). In contrast, phosphorothioate oligonucleotides of 15, 21, and 30 nucleotides in length and various sequences were stable for at least 24 hr when incubated with various cells. In studies in HeLa cells in which ISIS 1082, a 21-mer phosphorothioate, was incubated with the cells, then extracted from cells at various time points and analyzed on polyacrylamide gels, the compound was intact for four days (73).

Methylphosphonate oligonucleotides have also been shown to be stable in a variety of cells lines and extracts (14). No other class of oligonucleotides, however, has been sufficiently studied to allow definitive conclusions.

CELLULAR UPTAKE AND DISTRIBUTION Antisense oligonucleotides typically are 15–30 nucleotides long and thus have molecular weights that range from 4500–9000 daltons. The charge carried by phosphodiesters is, of course, negative and they are highly water soluble. The charge and hydrophilicity of modified oligonucleotides vary depending on the modifications. Consequently, membrane transport and cellular distribution are likely to vary widely as a function of the modifications introduced into oligonucleotides. For the two classes of modified oligonucleotides for which significant data have been reported—methylphosphonates and phosphorothioates—this is clearly the case. For both classes of oligonucleotides, the evidence is compelling that they do enter many cells at pharmacologically relevant concentrations.

Methylphosphonates are uncharged and lipophilic. Although thought to be taken up by most cells in tissue culture via passive diffusion, detailed studies of the kinetics of cellular uptake, distribution, and metabolism of uniformly labeled methylphosphonates have not been reported. Studies in Syrian hamster fibroblasts on oligonucleotides 3–9 nucleotides in length showed linear cell association for 1 hr, then reduced uptake. At equilibrium, the intracellular concentration of oligonucleotide was reported to be equivalent to the extracellular concentration (27, 74). In another study, a 21-mer methylphosphonate labeled with ^{32}P at the 5' terminus was reported to be taken up by CV-1 cells. Cell association was linear for 2 hr. Unfortunately, however, studies proving that the cell-associated radioactivity represented intact oligonucleotide were not presented. Nor were detailed studies on characteristics of uptake or intracellular distribution presented (75).

Phosphorothioates are negatively charged, but because of the sulfur atoms they may be slightly more lipophilic than phosphodiesters and tend to bind nonspecifically to serum proteins. Studies in our laboratories have shown that phosphorothioate oligonucleotides bind to serum albumin and that in the presence of serum albumin, cell-association is reduced (73; G. D. Hoke et al, unpublished observations).

Studies employing a 28-mer phosphorothioate deoxycytidine that was uniformly labeled with ^{35}S demonstrated that when HeLa cells were incubated with 1 μM of the drug, significant intracellular concentrations were achieved. Cellular uptake was linear, reaching a plateau of 60 p mole/10^6 cells in 6 hr. Adsorption to the cell membrane was minimal. Uptake was also concentration-dependent, reaching a plateau at approximately 1 μM. The drug associated with HeLa cells was intact for 24 hr and was located in both nuclei and cytoplasm. Infection with herpes simplex virus type 2, but not type 1, increased cellular uptake (76).

Studies in our laboratories have confirmed and extended the observations on phosphorothioate oligonucleotides. The cellular uptake, distribution, and metabolism of ISIS 1082, a uniformly ^{35}S labeled 21-mer phosphorothioate with a mixed antisense sequence, have been characterized in HeLa cells and HeLa S_3 cells, a variant line conditioned to growth in suspension. Incubation of HeLa cells with 5 μM of the drug resulted in approximately 8% of input radioactivity being associated with the cells. Cell association was linear for approximately 8 hr, and approximately 20% of the cell-associated radioactivity appeared to be adsorbed to the membrane. Uptake was temperature-dependent, required viable cells, and was inhibited by metabolic poisons. Uptake was concentration-dependent, and was linear to 10 μM. Uptake was influenced slightly by calcium and magnesium and was saturable. Natural oligonucleotides and methylphosphonates did not compete for uptake while other phosphorothioates competed; however, different length and sequence phosphorothioates competed differently (73, 77).

We have also studied other phosphorothioates of various lengths and other cell lines. HL 60 cells appear to take up less phosphorothiate oligonucleotides than HeLa cells and HeLa S_3 cells take up very little drug (73). Although not directly compared, human umbilical vein endothelial cells also appear to take up less drug than HeLa cells. Thus, there is considerable variation in the extent of uptake as a function of cell type.

In all cells studied, and with all uniformly labeled phosphorothioate oligonucleotides of varying size and sequences, we have shown that these drugs are stable in cells and cytoplasmic and nuclear extracts. In HeLa cells, no degradation of intracellular ISIS 1082 was observed for four days (73). Preliminary studies confirmed that these oligonucleotides distributed to both cytoplasm and nuclei and showed that there is an active temperature-dependent efflux process as well (77, 78).

When incubated with cells in the absence of serum or heat-inactivated serum, several laboratories have reported the apparent uptake of phosphodiester deoxyuligonucleotides. Moreover, a number of laboratories have reported activities for phosphodiester oligonucleotides that apparently were due to cellular uptake and intracellular activities. The studies on cellular uptake are not fully convincing, however Loke et al (79) studied deoxythymidine oligonucleotides ranging from 3–20 nucleotides in length and labeled with acridine at the 3' terminus. They incubated HL60 and three other hematopoietic cell lines with 12.5 μM of the acridine labeled drug and used flow-cytometric analyses of acridine fluorescence to quantitate cellular uptake. Uptake was reported to decrease as the length of the oligonucleotide increased and to vary as a function of the cell type. Uptake achieved a plateau in HL60 cells in 50 hr and was inhibited by polynucleotides of any length. The authors concluded that the oligonucleotides were taken up by an endocytotic mechanism. Unfortunately, the stability of the oligonucleotide-acridine conjugate was not rigorously documented. Nor were possible effects of acridine in the uptake of the oligonucleotide rigorously explored. Additionally, possible quenching or enhancement of the fluorescence of acridine by cellular interactions was not explored. Finally, extrapolations from homopolymers to mixed sequences have not been proven to be valid.

Another study employing phosphodiester oligonucleotides reached similar conclusions (80). Again, for most of the experiments, oligodeoxythymidines of 8 to 16 nucleotides in length were incubated with L929 mouse fibroblasts in the absence of serum. Maximal uptake occurred within 2 hr and upon incubation with fresh medium, cell-associated ^{32}P was released. Substantial degradation of the 5' labeled oligonucleotide was observed within 2 hr, and the authors concluded that approximately 20% of the radioactivity was in nuclei. Again, the authors concluded that the most likely mechanism of uptake was endocytosis (80).

Other pendant modifications of phosphodiester oligonucleotides have also been studied. A 9-mer labeled with acridine at the 3' terminus was reported to be taken up by *Trypanosome brucei* (61). More recently, the same group has reported that a 9-mer coupled at the 3' terminus to acridine via a dodecanal linker was more active in cells expressing mutated RAS than a 9-mer with a 3' acridine only (81). 3' poly-L-lysine-oligonucleotides have been reported to be stable to serum nucleases and to have enhanced activity as compared to phosphodiesters. Uptake was not studied, however, (63, 82, 83). In a later publication, the uptake of a poly-L-lysine oligonucleotide conjugate was enhanced compared to the unmodified oligonucleotide (84). When used to treat cells other than L929 cells, however, poly-L-lysine conjugates were inactive (64).

A number of lipid conjugates have also been studied. 5' linked triethylammonium 1,2 di-O-hexadicyl-rac-glycerol-3-H-phosphonate oligonucleotides were taken up 8–10-fold more than unmodified oligonucleotides by L929 cells and were more active against varicella zoster viral infections, albeit at high concentrations (85). An oligonucleotide linked at the 5' terminus to an undecyl residue was reported to be active, but no uptake or stability studies were reported (86).

The intracellular fate of oligonucleotides injected into oocytes and the uptake of oligonucleotides into oocytes have also been studied. When injected into *Xenopus* oocytes, unmodified oligonucleotides were degraded within 1 min primarily by 3' exonuclease digestion, but other nucleolytic activities were also present (87, 88). Interestingly, in this system, even phosphorothioate oligonucleotides were reported to be degraded, albeit much more slowly than phosphodiesters (89). These observations were extended in studies on oligodeoxynucleotides injected into CV-1 endothelial cells. A 28-mer oligonucleotide of either phosphodiester, phosphorothioate, or methylphosphonate type was injected into the cytoplasm of these cells. All three types of oligonucleotides localized to the nucleus in a temperature- but not energy-dependent fashion. The methylphosphonate oligonucleotide concentrated in regions of genomic DNA, in contrast to the two other oligonucleotides that co-localized with small nuclear ribonucleoproteins (90). Uptake of unmodified oligonucleotides by pre-implantation embryos was reported to be virtually nil (91).

Liposomes and related formulations have been shown to enhance cellular uptake of oligonucleotides in vitro. Loke et al (92) compared the uptake of phosphodiester and phosphorothioate deoxythymydine heptamers into HL-60 cells by using oligonucleotides coupled to 2-methyloxy-6-chloro 9-(5-hydroxypentyl) amino acridine and monitoring with flow cytometry. They did not determine the integrity of the oligonucleotides, but reached the conclusion that phosphodiester dT_7 was taken up by HL-60 cells much more effectively than phosphorothioate d-T_7, and that uptake plateaued at 50 hr. They reported increased anti-c-*myc* activity of phosphorothioate oligonucleotides after loading them in phosphatidyl serine liposomes. The uptake of a tetramer 2'-5' deoxyadenylate into L1210 cells was reported to be increased by loading the oligo-adenylate into *Staphyloccus aureus* protein A-crosslinked phospholipid vesicles (93). In our laboratories, we have shown that lipofectin, a cationic lipid mixture, can significantly increase the uptake and activity of phosphorothioate oligonucleotides in several cell lines. It also alters the intracellular distribution of these nucleotides (78).

With the exception of methylphosphonates, the conclusion from studies that have addressed the mechanisms of uptake of oligonucleotides is that the most likely mechanism is receptor-mediated endocytosis. In fact, in one study an 80-kd protein that appeared to bind oligonucleotides was partially purified

and postulated to be a "receptor" (79). The evidence supporting this mechanism is limited, however, and data are insufficient to conclude that receptor-mediated endocytosis is the most common or only mechanism of uptake of charged oligonucleotides in most cells.

In conclusion, although many questions remain to be answered, it appears that many cells in tissue culture may take up oligonucleotides at pharmacologically relevant concentrations. Clearly, oligonucleotides of different types behave differently and there are substantial variations as a function of cell type. Moreover, length and specific sequences may alter uptake, and pendant modifications may profoundly influence cellular uptake.

Once in the cell, it would seem that oligonucleotides distribute to the cytoplasm and the nuclei. In most if not all cells, phosphodiester oligonucleotides are rapidly degraded whereas methylphosphonates and phosphorothioates are much more stable. Again, pendant modifications may alter the rate of intracellular degradation and distribution.

Mechanisms of uptake and distribution are poorly understood. Clearly, however, multiple mechanisms may play a role, and different types of oligonucleotides may behave very differently.

Novel formulations may enhance cellular uptake. Liposomes and cationic lipids significantly enhance uptake and may alter the mechanisms of uptake and intracellular fate of oligonucleotides.

[redacted] Preliminary in vivo pharmacokinetic data are now available on methylphosphonate and phosphorothioate oligonucleotides. A 12-mer ^{3}H-labeled methylphosphonate injected in the tail vein of mice was rapidly cleared as intact oligonucleotide and distributed broadly to all tissues except the brain (94).

More extensive studies have been performed on ^{35}S-labeled phosphorothioates in rats. A true distribution phase of 15–25 min was observed after a single IV dose of a 27-mer followed by a prolonged elimination phase of 20–40 hr (94). The prolonged elimination phase may result from the binding of phosphorothioates to serum proteins. Phosphorothioates distrubuted broadly to all tissues except the brain and were eliminated in the urine intact. Phosphorothioates were rapidly and extensively absorbed after IM and IP administration (94).

Repeated daily doses of 50 mg/kg of a 27-mer phosphorothioate to mice resulted in similar distribution and elimination kinetics but slight differences in tissue concentrations from single dose studies. Liver, kidney, spleen, and lung were the organs with highest concentrations. Again, the drug was excreted intact in the urine (94).

Continuous osmotic pump administration of the same compound subcutaneously for 4 wk at doses of 50–150 mg resulted in similar pharmacokinetics (94).

Studies with ISIS 1082, a 21-mer phosphorothioate, in mice showed that when applied to the cornea in a sodium acetate buffer, significant adsorption to the cornea and absorption into the aqueous and vitreous humors occurred. Moreover, significant systemic bioavailability was observed (78). In rabbit, as much as 25% of an applied ocular dose was systemically bioavailable (unpublished observations). Post absorption pharmacokinetics were equivalent to IV pharmacokinetics.

Recently, a 20-mer phosphodiester was administered intravenously to rabbits. Clearance from blood was rapid and, after 90 min, 16% of the dose was found in the urine and was intact. In blood, at least 17% of the drug was estimated to be completely degraded within 5 min (95).

[redacted]

IN VITRO

Phosphodiesters Very little information has been published on the in vitro toxicities of unmodified oligonucleotides. In most systems, the oligonucleotides are thought to be rapidly degraded. When a 15-mer complementary to a c-myc sequence was incubated with human lymphocytes at 30 μM for 4 hr, no toxicity was observed. Longer incubation (24 hr) in 10% serum resulted in reduced ^{3}H-thymidine incorporation, but the authors concluded that this was probably due to dilution of the thymidine pool by thymidine liberated after rapid degradation of the oligonucleotide (96).

The incubation of a transformed leukemic cell line with 50 μM of a 20-mer complementary to a sequence in the BCL-1 proto-oncogene was reported to result in no decrease in viability as judged by trypan blue exclusion (97).

Methylphosphonates Incubation of Vero cells with 30 μM and lower concentrations of an 8-mer methylphosphonate for 24 hr resulted in no decrease in growth rate or cell count; however, 48 hr incubation resulted in 40% inhibition of growth rate (98). Similarly, neither of three 9-mers had any effect on L929 cell plating efficiency or protein synthesis after 16 or 40 hr incubations with 150 μM of drug (99). Incubation of T15 cells with 80 μM of a 9-mer directed against N-ras for 48 hr produced no effect on protein synthesis or viability (100). Similar results were reported for HT29 cells.

Inasmuch as methylphosphonate oligonucleotides have, when they have displayed activity, effective concentrations of 50–100 μM, the therapeutic index in vitro may be rather modest. Much more detailed studies are required before reaching final conclusions, however.

Phosphorothioates Phosphorothioate oligonucleotides bind to a variety of proteins, including serum albumin. In cell free protein translation experiments, they have been shown to induce nonspecific inhibition of protein synthesis (11, 101, 102). In wheat germ and rabbit reticulocyte lysate assays,

concentrations as high as 100 nM of a 17-mer phosphorothioate targeted to the protein mRNA inhibited globin synthesis relatively specifically. At 10 μM, nonspecific effects were observed (103). The nonspecific effects of phosphorothioates in these assays are length-dependent, as a 5-mer was much less potent than the 14-mers and dC28 appeared to be the most potent phosphorothioate oligonucleotide tested. In studies in our laboratories, we have made similar observations with a number of phosphorothioate oligonucleotides (G. D. Hoke et al, unpublished observations).

Phosphorothioate oligonucleotides have also been shown to inhibit DNA polymerases, reverse transcriptases, and nucleases when incubated in cell free systems (76, 77, 104).

Despite the potential nonspecific interactions of phosphorothioate oligonucleotides with cellular proteins, a wide variety of compounds have been shown to have excellent therapeutic indices. Microinjection of nanomolar concentrations of a 17-mer into *Xenopus* oocytes inhibited β-globin synthesis. When 16 μM of the compound were injected, however, protein synthesis was aborted and the oocytes underwent extensive cytolysis (89).

Incubation of cells in vitro with phosphorothioate oligonucleotides has likewise resulted in toxicities only at concentrations much higher than those at which therapeutic activities were observed. Human mononuclear cells were unaffected after 20 hr of incubation with 25 μM of several 15-mers (105). T697 cells were unaffected by a three-day exposure to 25 μM of a 20-mer (97, 106).

In our laboratories, we have determined the effects of ISIS 1082, a 21-mer phosphorothioate that inhibits herpes simplex virus types 1 and 2 infections in HeLa cells at 200–400 nM, on HeLa cell viability, DNA synthesis, RNA synthesis, protein synthesis, and energy metabolism. At no concentration below 500 μM were statistically significant effects observed after incubation for 96 hr. Exposure of HeLa cells to 500 μM ISIS for 48 hr resulted in 20% inhibition of protein synthesis (77). Similar results were observed in other cell lines.

Table 4 presents results from studies on 20 phosphodiester or phosphorothioate oligonucleotides targeted to various regions in the 5-lipoxygenase gene. Again, most of the phosphorothioates displayed toxicities only at 50 μM and greater. The exceptions to this rule were three 30-mers that inhibited cell growth at 10–35 μM. Clearly, one can conclude from this study that toxicity was time- and concentration-dependent and that, with longer exposures in particular, phosphorothioates were more toxic than their phosphodiester analogs (73).

We have identified other factors that influence the toxicity of phosphorothioates. Cell type may alter toxicity significantly. A comparison of the toxic effects of a 15-mer phosphorothioate on HL60 cells, U937 cells, and RBL-1

Table 4 In vitro toxicities of 5-lipoxygenase oligonucleotides in HL-60 cells[a]

Compound	Oligonucleotide Class[b]	Length	Sequence	AT:GC	24 hr	48 hr	72 hr	96 hr
1787	PD	15	5'-GTGTGCCACCAGCAG-3'	1:2	21.5	18.5	16.0	14.4
1788	PS	15			>100	35.0	25.0	19.0
1789	PD	30	AATGGTGAATCTCAC		>100	>100	>100	>100
1790	PS	30	GTGTGCCACCAGCAG	1:1.1	>100	>100	15.0	11.8
1795	PD	15	TGCCAGTGATTCATG	1:0.88	63.0	39.5	34.0	26.0
1796	PS	15			>100	>100	50.0	35.0
1797	PD	30	GGTCTTCCTGCCAGT		>100	>100	50.0	74.0
1789	PS	30	GATKATGACCCGCT	1:1.31	>100	20.0	10.0	10.0
1799	PD	15	GTCCTGATGGCTTCC	1:1.5	28.0	25.0	22.0	22.0
1800	PS	15			>50.0	50.0	50.0	34.0
1801	PD	30	GTCCTGATGGCTTCC		>50.0	>50.0	>50.0	>50.0
1802	PS	30	CACACCAGGAGCCCG	1:2.0	35.0	27.0	21.0	3.9
1812	PD	15	GTTCCTCCTTGGTGT	1:1.14	29.0	17.0	16.0	18.0
1813	PS	15			>50.0	32.0	25.0	43.0
1814	PD	30	ATTTGCTGTTGCTGC		>50.0	32.0	25.0	
1815	PS	30	TTCGTGTGGAAATGC	1:0.88	10.0	9.0	10.0	43.0
1816	PD	15	AGGTGTCCGCATCTA	1:1.14	150.0	250.0	>50.0	>50.0
1817	PS	15			32.0	>50.0	>50.0	>50.0
1818	PD	30	TCGGCGCGGCGGTCC	1:2.33	>50.0	>50.0	>50.0	>50.0
1819	PS	30	AGGTGTCCGCATCTA		15.0	19.0	18.0	20.0

[a] HL 60 cells were incubated in 96 well plates with increasing concentrations of oligonucleotides (1-50 or 100 μ M) in the presence of 10% fetal bovine serum. Viability of the cells was determined at each time point by trypan blue exclusion. IC_{50} values were obtained by plotting percentage control cell number vs. drug concentration.

[b] PD = Phosphodiester; PS = Phosphorothioate.

cells showed considerable variation in sensitivity; HL60 cells were the most sensitive. As phosphorothioates bind to serum albumin, in the presence of 10% fetal calf serum, a 15-mer produced no cytotoxicity after 24 hr of incubation at 100 μM. In the presence of 2.5% fetal calf serum, the IC_{50} was 19 μM. Finally, the purity of the oligonucleotide has a significant effect. Purification of oligonucleotides in triethyl ammonium buffers with trityl-on HPLC followed by removal of the trityl groups in triethyl ammonium may result in substantial contamination with triethyl ammonium ions, which are toxic to cells (73). Others have alluded to batch-to-batch variations and the potential that contaminants might contribute to toxicities, but they have not identified potential toxins (60, 97, 106–108).

Pendant group modified oligonucleotides Limited information is available concerning the effects of pendant groups on the toxicities of oligonucleotides. An acridine conjugated 7-mer phosphodiester was reported to produce no toxicities at 100 μM even though the free acridine had an IC_{50} for cell viability of 2 μM (109). Two 11-mer phosphorodiesters that were covalently attached to an undecyl group at the 5' terminus had no apparent toxic effect on MDCK cells at 100 μM (86). 5'terminal phospholipid conjugates of both phosphodiester and phosphorothioate oligonucleotides produced little toxicity in L292 cells when incubated at 50 to 100 μM (85). In contrast, a phosphodiester 15 mer linked to poly-L-lysine was toxic to L929 cells at 1 μM (84).

Table 5 summarizes published data concerning the in vitro toxicology.

[redacted] Although only preliminary toxicologic data are available, considerably more information should soon be available, as several compounds are currently in preclinical development.

Single-dose toxicity studies in mice were reported for phosphodiester (19), methylphosphonate (110), phosphomorpholidate, and phosphorothioate oligonucleotides. Unmodified oligonucleotides resulted in deaths in two of four treated mice at 160 mg/kg and all four mice treated with 640 mg/kg IV. Within three days after injection, a phosphorothioate oligonucleotide resulted in equivalent toxicities to the phosphodiester. The other analogs produced similar toxicologic effects with slight differences in doses.

Single doses of as much as 3.5 mg of a 27-mer complementary to the REV gene of HIV given IV or IP produce no toxicities in rats. Daily injections of 50 mg/kg IV of the same compound for 12 days in mice resulted in no observable toxicities. This 27-mer was also administered via a subcutaneous osmotic pump designed to administer up to 150 mg at a constant rate for 4 wk to rats. Again, no toxicities in any organ were observed (94).

ISIS 1082, 1 21-mer phosphorothioate targeted to inhibit herpes virus types 1 and 2, has been administered topically to mouse and rabbit eyes for as much as 21 days and resulted in no ocular toxicities. In rabbits, other organs were examined, and no effects were observed. Given the extensive bioavilability of ISIS 1082 in rabbits after ocular administration, this constitutes a significant observation.

Single doses of ISIS 2105, a 20-mer phosphorothioate active against human papilloma viruses, were administered intradermally and resulted in no local or systemic toxicities.

Consequently, a growing body of data supports the contention that at least single doses of phosphorothioate oligonucleotides may be given to mice, rats, and rabbits without significant acute or subacute toxicities.

MUTAGENICITY Virtually no data have been published on the potential mutagenicity of oligonucleotides. A 27-mer phosphorothioate was reported to be negative in an Ames assay in the presence or absence of a liver metabolic activation system at doses as high as 5 mg/plate (101).

P. Iverson (personal communication; 101) compared a number of oligonucleotide types and related chemicals in hamster lung fibroblasts. Unfortunately, although this study has been cited, the primary data have never been published, and thus it is difficult to draw any conclusion.

Mechanisms of Action of Oligonucleotides Interacting with Nucleic Acid Targets

The mechanisms by which interactions of oligonucleotides with nucleic acids may induce biological effects are complex and potentially numerous. Furthermore, very little is currently understood about the roles of various mechanisms or the factors that may determine which mechanisms are involved after oligonucleotides bind to their receptor sequences. Consequently, a discussion of mechanisms remains largely theoretical. Although a number of potential schemes to classify mechanisms of action might be employed, I prefer a scheme based on drug-receptor concepts.

OCCUPANCY-ONLY MEDIATED MECHANISMS Classic competitive antagonists are thought to alter biological activities because they bind to receptors, thereby preventing natural agonists from binding and inducing normal biological processes. Binding of oligonucleotides to specific sequences may inhibit the interaction of the RNA or DNA with proteins, other nucleic acids, or other factors required for essential steps in the intermediary metabolism of the RNA or its utilization by the cell.

Transcriptional arrest Oligonucleotides may bind to DNA and prevent either initiation or elongation of transcription by preventing effective binding of factors required for transcription, thus producing transcriptional arrest.

Table 5 In Vitro toxicology of antisense oligonucleotides

Oligonucleotide class	Length	Concentration	Target	Cell type	Time	Toxicity assessment	References
Phosphodiester	15	30 μM	c-*myc*	Human T cells	4 hours	Nontoxic	99
Phosphodiester	20	150 μM	βCL2	697 cells	3 days	Nontoxic	100
Phosphorothioate	20	25 μM	βCL2	697 cells	3 days	Nontoxic	
Phosphodiester	23	1-30 ng	Vg 1	Xenopus oocytes	2 days	Nontoxic @ low concentrations, i.e. <5 ng	92
Phosphorothioate	23	1–30 ng	Vg1	Xenopus oocytes	2 days	Toxic @ 15-30 ng	
Phosphodiester-acridine conjugate	7	50-100 μM	Type A influenza	MDCK	3 days	Nontoxic	112
Acridine alone		2 μM		MDCK	3 days	Toxic	
Methylphosphonate	9	150 μM	v s v	L929	16,40 hours	Nontoxic	102
Methylphosphonate	9	80 μM	N-*ras*	T15	48 hours	Nontoxic	103
Methylphosphonate	9	80 μM	N-*ras*	HT29	48 hours	Nontoxic	
Phosphodiester	14	1-25 μM	HIV	ATH8	1 days	Minor toxicity @ all conc. (<35%)	20
Methylphosphonate	14	1-25 μM			7 days	Minor toxicity @ all conc. (<27%)	
Phosphorothioate (heteropolymer)	14-28	1-25 μM			7 days	Nontoxic	
Phosphorothioate (homopolymer)		1-25 μM			7 days	Nontoxic	76
Phosphorothioate (homooligomer)	28	3-50μM	HSV-2	HeLa S_3	72 hours	Nontoxic	
Phosphorothioate (antisense)	20	4-100 μg/ml	HIV	H9, MOLT3	96 hours	Nontoxic	19
Phosphorothioate (nonsense)	20	4-100 μg/ml		H9, MOLT3	96 hours	Nontoxic (4 μg/ml) Toxic (20 μg–5% 100 μg-67%)	
Phosphodiester homooligomer (dT)	15	4-100 μg/ml		H9, MOLT3	96 hours	Toxic	
Phosphodiester homooligomer (dA)	15	4-100 μg/ml		H9, MOLT3	96 hours	Minor toxicity	
Phosphormorpholidate homooligomer (dT)	15	4-100 μg/ml		H9, MOLT3	96 hours	Toxic	
Phosphormorpholidate homooligomer (dG)	15	4-100 μg/ml		H9, MOLT3	96 hours	Toxic	
Phosphorothioate	17	16 μM	β-Globin	*Xenopus* oocytes	>6 hours	Toxic	106
Phosphorothioate	15	0.1–25 μM	IL1β	Human blood monocytes	20 hours	Nontoxic	108

It is possible that oligonucleotides could bind to segments of DNA that are partially denatured by the transcription complex, although this is highly unlikely. The initiation and elongation of transcription require a complex set of proteins and other factors, and it is difficult to conceive of a mechanism by which oligonucleotides might compete effectively against the transcriptional machinery for these single-stranded regions. Nevertheless, despite the improbability of such an event, reports of activities have been made that can be explained most simply by this mechanism (112, 113). Additionally, Helene and colleagues (114) reported that hexanucleotides to nonanucleotides with acridine derivatives at the 3' terminus inhibited transcription of the β-lactamase gene. When the RNA polymerase was preincubated with the oligonucleotide-acridine adducts, however, they observed nonspecific inhibition (115).

The alternative to seeking transient single-stranded regions or to attempting to denature a double-stranded region of DNA is to inhibit transcription by interacting with double-stranded DNA, i.e. forming triple-stranded structures. To form triple-stranded structures, hydrogen bonds other than Watson-Crick must be formed. In most current triple-strand motifs, the oligonucleotide becomes the third strand by recognizing hydrogen bonding donor/receptor sites on a purine reference strand and lying in the major groove (116–124). Alternative motifs have also been proposed. For example, Hogan and colleagues (125) proposed that a purine-rich oligonucleotide can form a triplex structure based upon the purines in the oligonucleotide base pairing in parallel fashion with the purines in the duplex DNA. Studies by Dervan's group (126), however, suggested that the purine-rich oligonucleotide bound to the duplex DNA with an antiparallel orientation.

The formation of triple-stranded structures by using natural nucleosides requires runs of purines Watson-Crick-hydrogen-bonded to their complementary pyrimidines. When cytidine is used to form a triple strand with a G-C base pair, it must be protonated; this occurs at nonphysiological acidic conditions (121). Furthermore, all motifs employ one or more "weak" hydrogen bonds. Thus, to achieve sufficient stability, relatively long triple-strand structures are required.

The principal theoretical advantage of triple helical inhibition schemes is that transcription represents the first step in the intermediary metabolism of RNA and may, therefore, provide substantial leverage for drug therapy. The other advantages that have been suggested are much more speculative. For example, it has been suggested that the smaller number of genes (one or two) compared to the number of mRNA molecules (usually less than 1000) per cell is an advantage for approaches that inhibit transcription. This suggestion ignores the kinetics of the targets, however. Genes have an infinite half-life relative to cell life. RNA molecules are synthesized and degraded with

varying kinetics. Furthermore, a variety of mechanisms exist to assure that even covalent modifications of DNA are repaired. Another concept has been that triple helixes in DNA might produce permanent biological effects. That even alkylating and DNA-cleaving anticancer drugs do not produce permanent effects points to the speciousness of this notion.

A number of theoretical disadvantages of triple helical inhibition of transcription have also been enumerated. Sequence specific binding is not yet possible, as runs of homopyrimidines are required. These sequences may play important regulatory roles in DNA, as they are much more abundant than statistically predicted (16). Longer term, a more substantial problem may simply be gaining sequence-specific access to DNA in chromatin. Additionally, deliberate interactions with the genome raise concerns about mutagenicity, carcinogenicity, and teratogenicity, which, in most therapeutic settings, are of considerable importance.

Several strategies have been developed to circumvent the requirement for purine-pyrimidine runs and other limitations. For example, purine oligonucleotides form triplex structures at higher pH values than pyrimidine-rich oligonucleotides (125, 126). Similarly, pyrimidine-rich oligonucleotides, in which 2'-O-methyl pseudoisocytidine was substituted for 2' deoxycytidine, formed triplex structures as neutral pH (127). Oligonucleotides with linkers that allow crossover of the oligopyrimidine from one strand of the duplex to the other have been reported and this motif suggested to be a solution to a broader sequence repertoire (128). To enhance the stability of triple helices, intercalators and photoactivatable crosslinkers and alkylators have been conjugated to oligo pyrimidines (129–131). To increase potency and enable identification of sites of binding, a number of cleavage moieties have been conjugated to oligopyrimidines (132–137). Finally, to enhance nuclease stability, methylphosphonates (138) and α-oligonucleotides (136) have been shown or suggested to form triple helices.

In addition to cleavage of DNA in vitro by triplex-forming oligonucleotides coupled to cleavage reagents and alkylation induced by oligonucleotide-coupled alkylators, several other methods have been used to show triplex formation. These include agarose affinity column purification (139), NMR (140), protection from uv dimerization (141), solution hybridization (142), inhibition of binding of DNA-binding proteins (143), inhibition of restriction endonucleases (144), and repression of c-myc transcription in vitro (125). Recently, a 28-mer phosphodiester stabilized at the 3' end by alanine and directed to enhancer elements for the IL-2 receptor gene was shown to inhibit the transcription of the gene when incubated with human lymphocytes. The authors reported evidence for selectivity to oligonucleotides as well (145).

Obviously, triple-helix-based inhibition of transcription is of potential therapeutic importance, particularly for targets that for a variety of reasons

may be difficult to inhibit at the post-transcriptional level. Substantial medicinal chemistry must be completed, however, to create oligonucleotides that can interact with duplex structures in a sequence-specific fashion without requiring special motifs. Once this is accomplished, of course, additional studies must show that the other theoretical limitations discussed above can be overcome.

Inhibition of splicing A key step in the intermediary metabolism of most mRNA molecules is the excision of introns. These "splicing" reactions are sequence-specific and require the concerted action of spliceosmes. Consequently, oligonucleotides that bind to sequences required for splicing may prevent binding of necessary factors or physically prevent the required cleavage reactions. This then would result in inhibition of the production of the mature mRNA. Although there are several examples of oligonucleotides directed to splice junctions, none of the studies presented data showing inhibition of RNA processing, accumulation of splicing intermediates, or a reduction in mature mRNA. Nor are there published data in which the structure of the RNA at the splice junction was probed and the oligonucleotides demonstrated to hybridize to the sequences for which they were designed (146–149). Activities have been reported for anti-c-*myc* and antiviral oligonucleotides with phosphodiesters, methylphosphonates, and phosphorothioates.

Translational arrest Without question, the mechanism for which the majority of oligonucleotides have been designed is translational arrest. Oligonucleotides have been designed to bind to the translational initiation codon. The positioning of the initiation codon within the area of complementarity of the oligonucleotide and the length of the oligonucleotide used have varied considerably. Again, unfortunately, only in relatively few studies have the oligonucleotides been shown to bind to the sites for which they were designed, and other data that support translation arrest as the mechanism reported.

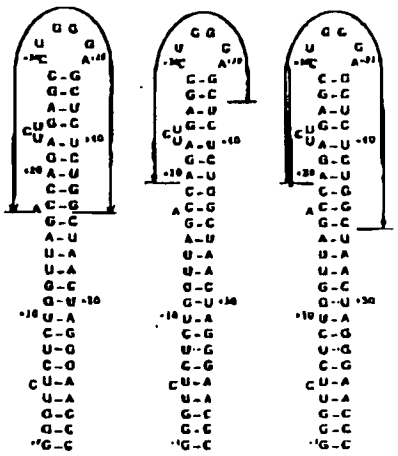
Target RNA species that have been reported to be inhibited include HIV (19), vesicular stomatitus virus (VSV) (82), n-*myc* (150), and a number of normal cellular genes (151–154).

In our laboratories, we have shown that a significant number of targets may be inhibited by binding to translation initiation codons. For example, ISIS 1082 hybridizes to the AUG codon for the UL13 gene of herpes virus types 1 and 2. Studies with RNaseH confirmed that ISIS 1082 binds selectively in this area. In vitro protein synthesis studies confirmed that ISIS 1082 inhibited the synthesis of the UL13 protein, and studies in HeLa cells showed that it inhibited the growth of herpes type 1 and type 2 with an IC_{50} of 200–400 nm

by translation arrest (155). Similarly, ISIS 1753, a 30-mer phosphorothioate complementary to the translation initiation codon and surrounding sequences of the E2 gene of bovine papilloma virus, was highly effective, and its activity was shown to be due to translation arrest. ISIS 2105, a 20-mer phosphorothioate complementary to the same region in human papilloma virus, was shown to be a very potent inhibitor. Compounds complementary to the translation initiation codon were the most potent of the more than 50 compounds studied complementary to various other regions in the RNA (156).

In conclusion, translation arrest represents an important mechanism of action for antisense drugs. A number of examples purporting to employ this mechanism have been reported. Recent studies on several compounds have provided data that unambiguously demonstrate that this mechanism can result in potent antisense drugs.

Disruption of necessary RNA structure RNA adopts a variety of three-dimensional structures induced by intramolecular hybridization, the most common of which is the stem loop (Figure 2). These structures play crucial roles in a variety of functions. They are used to provide additional stability for



length	28	18	26
P=S	1972	1308	1307
P=O	1971	1116	1118

Figure 2 Antisense oligonucleotides directed against the HIV TAR element. The oligonucleotide sequences are complementary to the tar sequences where indicated.

RNA and as recognition motifs for a number of proteins, nucleic acids, and ribonucleoproteins that participate in the intermediary metabolism and activities of RNA species. Thus, given the potential general activity of the mechanism, it is surprising that occupancy-based disruption RNA has not been more extensively exploited.

As an example, we designed a series of oligonucleotides that bind to the important stem-loop in all RNA species in HIV, and TAR element. We synthesized a number of oligonucleotides designed to disrupt TAR, and showed that several indeed did bind to TAR, disrupt the structure, and inhibit TAR-mediated production of a reporter gene (157). Futhermore, general rules useful in disrupting stem-loop structures were developed as well.

Although designed to induce relatively nonspecific cytotoxic effects, two other examples are noteworthy. Oligonucleotides designed to bind to a 17 nucleotide loop in *Xenopus* 28S RNA, required for ribosome stability and protein synthesis, inhibited protein synthesis when injected into *Xenopus* oocytes (158). Similarly, oligonucleotides designed to bind to highly conserved sequences in 5.8S RNA inhibited protein synthesis in rabbit reticulocyte and wheat germ systems (159).

OCCUPANCY-ACTIVATED DESTABILIZATION RNA molecules regulate their own metabolism. A number of structural features of RNA are known to influence stability, various processing events, subcellular distribution, and transport. As RNA intermediary metabolism is better understood, many other regulatory features and mechanisms will probably be identified.

5' Capping A key early step in RNA processing is 5' capping (Figure 1). This stabilizes pre-mRNA and is important for the stability of mature mRNA. It also is important in binding to the nuclear matrix and nuclear transport of mRNA. As the structure of the cap is unique and understood, it presents an interesting target.

Several oligonucleotides that bind near the cap site have been shown to be active, presumbly by inhibiting the binding of proteins required to cap the RNA. Again, however, this putative mechanism has not been rigorously demonstrated in any published study. In fact, none of the oligonucleotides have been shown in any published study to bind to the sequences for which they were designed. For example, the synthesis of SV40 T-antigen was reported to be most sensitive to an oligonucleotide linked to polylysine and targeted to the 5' cap site of RNA (160).

In studies in our laboratory, we have designed oligonucleotides to bind to 5' cap structures and reagents to specifically cleave the unique 5' cap structure (161).

Inhibition of 3' polyadenylation In the 3' untranslated region of pre-mRNA molecules, there are sequences that result in the post-transcriptional addition of long (hundreds of nucleotides) tracts of polyadenylate. Polyadenylation stabilizes mRNA and may play other roles in the intermediary metabolism of RNA species. Theoretically, interactions in the 3' terminal region of pre-mRNA could inhibit polyadenylation and destabilize the RNA species. Although there are a number of oligonucleotides that interact in the 3' untranslated region and display antisense activities, to date no study has reported evidence for alterations in polyadenylation.

Other mechanisms In addition to 5' capping and 3' adenylation, clearly other sequences in the 5' and 3' untranslated regions of mRNA affect the stability of the molecules. Again, a number of antisense drugs may work by these mechanisms.

Zamecnik & Stephenson (1) reported that a 13-mer targeted to untranslated 3' and 5' terminal sequences in Rous sarcoma viruses was active. Oligonucleotides that were conjugated to an acridine derivative and targeted to a 3'-terminal sequence in type A influenza viruses were reported to be active (109, 162, 163). Against several RNA targets, studies in our laboratories have shown that sequences in the 3' untranslated region of RNA molecules are often the most sensitive. For example, ISIS 1939, a 20-mer phosphorothioate that binds to and appears to disrupt a predicted stem-loop structure in the 3' untranslated region of the mRNA for ICAM, is a potent antisense inhibitor. However, inasmuch as a 2'-O-methyl analog of ISIS 1939 was much less active, it is likely that in addition to destabilization to cellular nucleolytic activity, activation of RNase H (see below) is also involved in the activity of ISIS 1939 (164).

ACTIVATION OF RNase H RNase H is an ubiquitous enzyme that degrades the RNA strand of an RNA-DNA duplex. It has been identified in organisms as diverse as viruses and human cells (for review see 165). At least two classes of RNase H have been identified in eukaryotic cells. Those in yeast and multiple enzymes with RNase H activity have been observed in prokaryotes (165). Furthermore, data suggest that there are multiple isozymes in eukaryotic cells.

Although RNase H is involved in DNA replication, it may play other roles in the cell and is found in the cytoplasm as well as the nucleus (166). The concentration of the enzyme in the nucleus is thought to be greater, however, and some of the enzyme found in cytoplasmic preparations may be due to nuclear leakage.

RNase H activity is quite variable. It is absent or minimal in rabbit reticulocytes (167) but present in wheat germ extracts (165) in a wide range of

cells (16). The level of RNase H varies as a function of development, differentiation, and rate of cell division (165). In HL60 cells, for example, the level of activity in undifferentiated cells is greatest; it is relatively high in DMSO and vitamin D–differentiated cells, and much lower in PMA-differentiated cells (G. D. Hoke et al, unpublished observations).

The precise recognition elements for RNase H are unknown; however, it has been shown that oligonucleotides with DNA-like properties as short as tetramers can activate RNase H (168). Changes in the sugar influence RNase H activation, as sugar modifications that result in RNA-like oligonucleotides, e.g. 2'-fluoro or 2'-O-methyl, do not appear to serve as a substrate for RNase H (44, 169). Alterations in the orientation of the sugar to the base can also affect RNase H activation, as α-oligonucleotides are unable to induce RNase H or may require parallel annealing (41, 170). Additionally, backbone modifications influence the ability of oligonucleotides to activate RNase H. Methylphosphonates are not substrates for RNase H (74, 143). In contrast, phosphorothioates are excellent substrates (106, 155, 171; G. D. Hoke unpublished observations). More recently, chimeric molecules have been studied as substrates for RNase H (172, 173). A single ribonucleotide in a sequence of deoxyribonucleotides was recently shown to be sufficient to serve as a substrate for RNase H when bound to its complementary deoxyoligonucleotide (174).

Despite the information about RNase H and the demonstrations that many oligonucleotides may activate RNase H in lysate and purified assays (168, 175–177), relatively little is known about the role of structural features in RNA targets in activating RNase H. There is little direct proof that RNase H activation is, in fact, the mechanism of action of oligonucleotides in cells. Recent studies in our laboratories provide additional, albeit indirect, insights into these questions. ISIS 1939 is a 20-mer phosphorothioate complementary to a sequence in the 3' untranslated region of ICAM-1 RNA. It inhibits ICAM production in human umbilical vein endothelial cells, and northern blots demonstrate that ICAM-1 mRNA is rapidly degraded. A 2'-O-methyl analog of ISIS 1939 displays higher affinity for the RNA than the phosphorothioate, is stable in cells, but inhibits ICAM-1 protein production much less potently than ISIS 1939. It is likely that ISIS 1939 destabilizes the RNA and activates RNase H. In contrast, ISIS 1570, an 18-mer phosphorothioate that is complementary to the translation initiation codon of the ICAM-1 message, inhibited production of the protein but caused no degradation of the RNA. Thus, two oligonucleotides that are capable of activating RNase H had different effects, depending on the site in the mRNA to which they bound (164).

COVALENT MODIFICATION OF THE TARGET NUCLEIC ACID BY THE OLIGONUCLEOTIDE A large number of oligonucleotides conjugated to alkylating and photoactive alkylating species have been synthesized and tested for effects on purified nucleic acids and intracellular nucleic acid targets (162, 178). The potential advantage of such modifications is, of course, enhanced potency. The potential disadvantages are equally obvious: nonspecific alkylation in vivo and resulting toxicities.

A variety of alkylating agents have been used to modify single-stranded DNA covalently and have been shown to induce alkylation at sequences predicted by the complementary oligonucleotide to which they were attached (178–182). Similar alkylators have been employed to modify double-stranded DNA covalently after triplex formation (125, 137, 183, 184).

Photoactivatable crosslinkers and platinates have been coupled to oligonucleotides and shown to crosslink sequence-specifically as well. Photoactivatable crosslinkers coupled to phosphodiesters, methyl-phosphonates, and phosphorothioates have been shown to produce sequence-specific crosslinking (59, 130, 185–190). Photoreactive crosslinking has also been demonstrated for double-stranded DNA after triplex formation (136, 191).

Preliminary data suggesting that covalent modifications of nucleic acids in cells is feasible and may enhance the potency of oligonucleotides have also been reported. Psoralen-linked methylphosphonate oligonucleotides were reported to be significantly more potent than methylphosphonate oligonucleotides in inhibiting rabbit globin mRNA in rabbit reticulocyte lysate assay (33). Psoralen-linked methylphosphonates were also reported to be more potent in inhibiting herpes simplex virus infection in HeLa cells in tissue culture (147). Additionally, although it did not produce covalent modification, a 9-mer phosphodiester conjugated with an intercalator inhibited mutant Ha-ras synthesis in T-24 bladder carcinoma cells (81).

OLIGONUCLEOTIDE-INDUCED CLEAVAGE OF NUCLEIC ACID TARGETS Another attractive mechanism by which the potency of oligonucleotides might be increased is to synthesize derivatives that cleave their nucleic acid targets directly. Several potential chemical mechanisms are being studied, and positive results have been reported.

The mechanism that has been most broadly studied is to conjugate oligonucleotides to chelators of redox-active metals and generate activated oxygen species that can cleave nucleic acids. Dervan and colleagues have developed EDTA-conjugated oligonucleotides that cleave double-stranded DNA sequence specifically after triplex formation (124, 137). Dervan and others have employed EDTA-oligonucleotide conjugates to cleave single-stranded DNA (192, 193). It is thought that EDTA chelates iron, which generates hydroxyl radicals that cleave the DNA; however, the cleavage occurs at several nucleotides near the nucleotide at which EDTA is attached.

In the presence of copper, oligonucleotides that are conjugated to 2,10-phenantholine also cleave DNA with some sequence specificity (129,

133–135, 194, 195), as do porphyrin-linked oligonucleotides when exposed to light (196–198). Phorphyrin-linked oligonucleotides, however, oxidize bases and induce crosslinks as well as cleave the phosphodiester backbone.

To date, no reports have demonstrated selective cleavage of an RNA or enhanced potency of oligonucleotides in cells using oligonucleotides and cleaving moieties that employ these mechanisms. Studies in progress in a number of laboratories will probably soon explore this question.

Another mechanism that may be intrinsically more attractive for therapeutic applications, particularly for cleavage of RNA targets, is a mechanism analogous to that used by many ribonucleases, nucleotodiyltransferases, phosphotransferases, and ribozymes.

Ribozymes are oligoribonucleotides or RNA species capable of cleaving themselves or other RNA molecules (199). Furthermore, the Tetrahymena ribozyme has been shown to cleave DNA, but at a slower rate than RNA (200). Although several classes of ribozymes have been identified that differ with regard to substrate specificity, the use of internal or external guanosine, and other characteristics, they all employ similar enzymatic mechanisms. Cleavage and ligation involve a Mg^{2+}-dependent transesterification with nucleophilic attack by the 3'-hydroxyl of guanosine (200).

The notion that we might design a relatively small ribozyme that could interact with desired sequences as a therapeutic was given impetus by studies that showed activity for ribozymes as short as a 19-mer (201) and by the demonstration that ribozyme activity can be retained after substitutions such as phosphorothioates are introduced (200).

Other approaches to creating oligonucleotides that cleave RNA targets are to synthesize oligonucleotides with appropriate adducts positioned to catalyze degradation via acid-base mechanisms. Substantial progress is being made in this area as well (P. D. Cook et al, unpublished observations).

A few studies have attempted to compare activities of oligonucleotides targeted to different receptor sequences in the same RNA. In our laboratories, we have shown that the most sensitive site in ICAM mRNA appears to be the 3' untranslated region (164). Against PLA_2, the most active molecules are also directed to sequences in the 3' untranslated region. In contrast, the most active molecules against ELAM are in the 5' untranslated region (C. F. Bennett et al, unpublished observations). However, oligonucleotides directed to the 5' cap site, translation initiation codon, and coding regions have also shown activity (for review see 202, 203).

In conclusion, an array of potential post-binding mechanisms have already been identified for oligonucleotides. For specific oligonucleotides, however, insufficient data are available to draw firm conclusions about mechanisms. More than one mechanism may very well play a role in the activity of a given oligonucleotide. Many additional mechanisms are likely to be identified as progress continues. It is important to consider the structure and function of receptor sequences in designing oligonucleotides and to continue to study potential mechanisms in detail. Clearly, RNase H may play a role in the mechanisms of many oligonucleotides, but, equally clearly, it is not critical for the activity of others. In the future, the mechanisms for which oligonucleotides are designed will probably be optimized for each target and class of oligonucleotide.

MEDICINAL CHEMISTRY

The core of any rational drug discovery program is medicinal chemistry. Although the synthesis of modified nucleic acids has been a subject of interest for some time, the intense focus on the medicinal chemistry of oligonucleotides dates perhaps to no more than three years prior to this writing. Consequently, the scope of medicinal chemistry has recently expanded enormously, but the biological data to support conclusions about synthetic strategies are only beginning to emerge. As several excellent reviews have been published recently, I focus here strictly on design features and progress in evaluating various approaches to enhance the properties of oligonucleotides as drugs (12, 16, 202, 203).

Modifications in the base, sugar, and phosphate moieties of oligonucleotides have been reported. The subject of medicinal chemical programs include approaches to create enhanced affinity and more selective affinity for RNA or duplex structures; the ability to cleave nucleic acid targets; enhanced nuclease stability, cellular uptake, and distribution; and in vivo tissue distribution, metabolism and clearance.

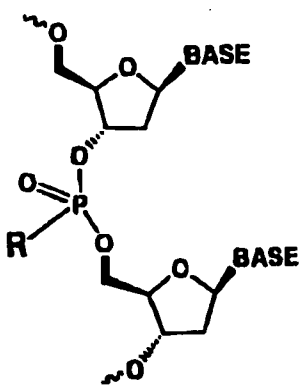
Modifications of the Phosphate

Table 6 shows the structures of various phosphate analogs. The properties of phosphodiester, phosphorothioate, and methylphosphonate analogs have been discussed extensively. More recently, phosphorodithioates have been synthesized and reported to be nuclease-resistant and to form stable duplexes with complementary DNA (204). Of course, a potential advantage of the phosphorodithioates is the lack of a chiral center. Another interesting recent modification is the replacement of one of the nonbonding oxygen atoms with a borane group (205). A dimer was reported to be nuclease-resistant, but little additional information is available.

Modifications of the Pentofuranose Linker

Modifications of oligonucleotides that replace phosphorous may be attractive because they support the design of oligonucleotides that may have a range of

Table 6 Phosphate modifications and analogs



Structure	R	Name
1	O^-	Phosphodiester
2	S^-	Phosphorothioate
3	Me	Methyl phosphonate
4	N (alkyl)	Phosphoramidate
5	S and O→S	Phosphorodithioate
6	H_3B	Boranophosphate

charges from nonionic to negatively or positively charged. In theory, reducing the anionic character of oligonucleotides may enhance hybridization and pharmacokinetic properties.

As discussed above, earlier modifications were not specifically directed to the needs of oligonucleotide drugs. Recent modifications have attempted to maintain the geometry and spacing required to support hybridization. Replacement of the phosphorous dioxygen moiety with a methylene group has been reported by two groups (51–53). This "formacetal" linkage forms a stable duplex with DNA and to be nuclease-resistant, but it is not amenable to additional modifications without creating chiral centers and would result in a water-insoluable molecule if fully substituted throughout an oligonucleotide. Other one-for-one substitutions have been reported, but the substitutions are either less interesting or have not been evaluated sufficiently to determine their potential (for review see 12).

More complex substitutions have also been reported recently, including two atom substitutions in which the phosphorous and 5' oxygen atoms were replaced by a sulfonamide linkage (54) or methyl sulfoxyl linkage (206). A thymine tetramer in which the phosphate backbone structure was replaced with dimethylene sulfonate was recently found to hybridize with natural oligonucleotides (207). Additionally, an acetamide group has been substituted for the backbone phosphate structure in a dinucleoside (207). Finally, a great many other substitutions in the backbone have been made and will probably be published in the next year, so the repertoire of compounds should continue to increase.

Pentofuranose (Sugar) Modifications

A significant number of modifications have recently been reported. In essence, these derive from two strategies with different objectives. Oligonucleotides in which the sugars are modified uniformly throughout are designed to enhance affinity to RNA targets by facilitating the formation of a more stable helix. They also may enhance nuclease stability and membrane permeability, but these outcomes are usually secondary to the hybridization goals. In contrast, pendant modifications have also been synthesized primarily to enhance pharmacokinetics or to introduce alkylating or cleaving moieties. In any event, the sugar at the 2' position is clearly an attractive site for medicinal chemistry. Some of the properties of a few of the 2' modified oligonucleotides are shown in Table 3. This remains a fertile area for medicinal chemistry, and additional advances are likely.

More substantive alterations in sugar and even replacement of sugar are also possible. Of course, α-oligonucleotides represent one type of modification (41), but numerous other approaches are feasible. Carbocyclic (49, 50) and acyclic (47, 48) structures have been reported.

Heterocyclic Modifications

Numerous heterocyclic modifications have also been described. Many of these have been designed to enhance affinity and/or alter specificity (224). Other modifications have been developed to attach pendant modifications that may alkylate, intercalate, or cleave, as well as others that may enhance pharmacokinetic properties.

Conclusions

In conclusion, it is clear that an enormous scope for medicinal chemistry exists and that the major programs are already beginning to pay dividends.

ACTIVITIES OF OLIGONUCLEOTIDES

In the past several years, scores of articles have been published demonstrating the activity of a large number of oligonucleotides in a variety of systems. A number of excellent reviews have summarized the activities of these compounds (16, 202, 203, 208). The activities of oligonucleotides in assays of cell-free protein synthesis and after injection into cells of several types have also been summarized. Consequently, I provide a brief summary of the activities of oligonucleotides in cell-based assays and a comment on the limited in vivo data reported to date.

Activities in Cells in Tissue Culture

To date, oligonucleotides have been found to inhibit the growth of a large number of viruses in tissue culture, the expression of numerous oncogenes, a variety of normal cellular genes, and a number of transfected reporter genes controlled by several regulatory elements. The oligonucleotides used, the cells employed, and the receptor sequences, concentrations, and conditions have differed widely. Only a few of the studies have reported detailed dose response curves and conditions. Studies for which sufficient information was presented are summarized in Table 7.

The data presented in Table 7 support only a few generalizations, as follows:

1. Even though phosphodiesters are relatively rapidly degraded, a number of laboratories have reported activities for unmodified phosphodiester oligonucleotides in cells incubated in the absence of serum. The concentrations required to display activity were typically greater than 10 μM.
2. A variety of modified oligonucleotides have been reported to be active. Methylphosphonates appear to be less potent than phosphorothioates, but considerable variation has been noted, depending on the system. Conjugation of alkylators and intercalators to phosphodiesters and methylphosphates increases potency. Lipophilic and poly-lysine conjugates have also displayed enhanced activities.
3. Oligonucleotides have demonstrated a broad array of activities against viral targets, oncogenes, normal host gene products, and various transfected genes. Thus, clear evidence supports the broad, potential applicability of these drugs.
4. Although the data from studies incorporated in Table 7 are limited, when combined with the in vitro toxicologic data, the therapeutic indexes of phosphorothioates appear to be quite high in vitro. Methylphosphonates appear to have lower therapeutic indexes. Too few data are available to draw conclusions about other classes of oligonucleotides.
5. Very few data support putative mechanisms of action, and generalizations concerning desired mechanisms of action are not possible. Nevertheless, a variety of mechanisms of action may be employed by oligonucleotides to result in significant biological activities.

In Vivo Activities

Two earlier investigations have suggested in vivo activities of antisense drugs against viral infections. Although no data were reported, a methylphosphonate oligonucleotide was indicated to have been active in a mouse model of herpes simplex virus I infection (147). Another study claimed in vivo activity against tick-borne encephalitis virus (213).

Table 7 Summary of antisense oligonucleotide Activities (in vitro)[1]

Target	Cell type	Serum	Oligo types	Length	Concentration	Reference
Viruses						
HTLV-III	H9 cells	–	P	12–26	5-50 mg/ml	146
LAV	H-T cells	+	PS	14–28	0.5 1 μM	20
HIV (gag/pol)	H-T cells	+	PS	18-24	1–10 μM	211
HIV	H9 cells	+	PS, others	20	4-20 μg/ml	19
HIV	CZM cells	+	PS	18–28	10 μM	157
Herpes simplex	Vero cells	+	CH_3P	7	50-100 μM	98
Herpes simplex	HeLa cells	+	PS	28	1–10 μM (non-antisense)	76
Herpes simplex	Vero cells	+	CH_3P	12	20-50 μM	15
Herpes simplex	Vero cells	+	CH_3P psoralen	12	5 μM	15
Herpes simplex	HeLa cells	+	PS	21	0.2-4 μM	155
Vesicular stomatitis	L929 cells	+	CH_3P	9	25-50 μM	99
Vesicular stomatitis	L929 cells	+	P-lipid	11	50-150 μM	85
Vesicular stomatitis	L929 cells	+	P-poly l-lysine	10–15	0.1 μM	212
Influenza	MDCK cells	+	P-acridine	11	50 μM	109
Tick-borne encephalitis	—	+	Various	Var.	0.1-1 μM	213
SV40	MDCK cells	+	CH_3P	6-9	25 μM	214
Rous	Chicken fibroblasts	+	Various	Var.	10 μM	1
Hepatitis B	Alexander	+	P	15	8.5 μM	215
Bovine papilloma virus	C-127 cells	+	PS	4-30	0.01-1 μM	209
Oncogenes						
c-*myc*	T-lymphocytes	+	P	15	30 μM	96
c-*myc*	HL-60 cells	+	P, PS	15	10 μM	216
c-*myc*	Burkitt cells	–	P	21	100 μM	146

c-*myb*	PMBC	+	P	18	40 mg/ml	217
BCL-2	L697 cells	−	P, PS	20	25–150 μM	97
N-*myc*	Neuroblastoma cells	+	P	15	1–5 μM	155
N-*ras*	T15 cells	+	CH_3P	9	Inactive	100
Host genes						
Multiple drug Resistance	MCF-1 cells	+	PS	15		218
PCNA (cyclin)	3T3	+	P	18	30 μM	219
Prothymosin	Human myeloma cells		P	22		152
T cell receptor	T cells	+	P	22		153
Gm CSF	Endothelial cells	−	P	15, 18	10^{-9}M	153
CSF-1	FL-*ras*/*myc* cells	+	P	?	?	220
EGF receptor		+	P	13	30 μM	221
BFGF	Human astrocytes	−	P	15	10-75 μM	229
β Globin	Rabbit reticulocytes	+	CH_3P	9	100 μM	222
TAU	Neurons	−	P	20-25	3-50 μM	228
CAMP-Protein kinase II β	HL-60 cells	+	P	21	15 μM	210
Myeloblastin	HL-60 cells	+	P	18	?	227
Phospholipase A_2 activating protein	$BC3H_1$	+	P	25	25 μM	235
ICAM-1	A549 HVEC lymphocytes	−	PS	18-20	0.01-1 μM	154
IL-2	T-lymphocytes	−	P	15	5 μM	72
IL-1α	HUVEC	+	P	18	10 μM	154
IL-1β	Monocytes	+	PS	15	0.1-2.5 μM	105

Table 7 (*Continued*)

Target	Cell type	Serum	Oligo types	Length	Concentration	Reference
IGF-1	Myoblasts	−	P	15	10 μM	225
Perforin	T-lymphocytes	−	P	18	5-35 μM	226
Other						
Chloramphenicol acetyl transferase	CV-1 cells	+	P, PS, CH_3P	21	5-30 μM	75
Placental alkaline phosphatase driven by HIV TAR	SK-mel-2 cells	+	PS	18-28	0.25-5 μM	157
Chloramphenicol Acetyl transferase Driven by human papilloma virus E2 responsive element	C-127 and CV-1 cells	+	PS	14–20	1–10 μM	209

[a] Abbreviations: cAMP, cyclic AMP; CH_3-P, methylphosphonate oligonucleotides; EGF, epidermal growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; HB, hepatitis B; HIV, human immunodeficiency virus; HSV, herpes simplex virus; HTLV, human T-cell lymphotrophic virus; IV, influenza virus; P, phosphodiester oligonucleotides; P-acridine, phosphodiester oligonucleotide conjugated with acridine moiety; P-lipid, phosphodiester oligonucleotide conjugated with lipid moiety; P-S, phosphorothioate oligonucleotides; PCNA, proliferating cell nuclear antigen; PMA, phorbol myristeric acid; RSV, Rous sarcoma virus; TAR, Tat response element; TBE, tick-borne encephalitis; VSV, vesicular stomatitis.

Topical application of ISIS 1082 in an aqueous buffer to the cornea of mice infected with herpes virus I inhibited viral growth in a concentration-dependent fashion and cured the infection at concentrations greater than 1% (230). The activity of ISIS 1082 was equivalent to trifluorothymidine and was associated with no local or systemic toxicities.

CONCLUSIONS

Oligonucleotides designed to interact with nucleic acid receptors represent a potentially revolutionary advance in pharmacotherapy. Advances in the recent past and the intense, current focus assure that the paradigm will be fully explored.

Oligonucleotides have already been shown to work in vitro and have proven to be invaluable pharmacologic tools. The progress in resolving the basic pharmacological questions relating to oligonucleotide therapeutics and in resolving issues that will influence the commercialization of new drugs of this class has been impressive. Moreover, advances in medicinal chemistry are exciting and argue that exciting new classes of these drugs are forthcoming.

Much remains to be learned; a great deal remains to be accomplished before the paradigm is fully proven and the opportunity it represents realized. In the coming years, the key tasks will be (*a*) to place oligonucleotide therapeutics on a solid pharmacological footing by performing careful dose response curves in well-designed experiments, and (*b*) to advance the development of oligonucleotide pharmaceutic products to the point that the paradigm can be tested in man.

There is now cause for considerable optimism that the promise of oligonucleotide therapeutics may be realized.

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PHARMACOLOGY OF PROTEIN KINASE INHIBITORS

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KEY WORDS: cAMP-dependent protein kinase, cGMP-dependent protein kinase, protein kinase C, Ca^{2+}/calmodulin kinase II inhibitor, protein phosphorylation

INTRODUCTION

A complete understanding of the organization and functioning of the second messenger system requires the expertise and cooperation of several different scientific disciplines, such as molecular pharmacology, genetic manipulation, biochemistry, and cell biology. The advent of a new class of effective pharmacological agents is always an event of considerable interest, in particular when this class consists of new types of antagonists that act by specifically blocking one or more of the steps in intracellular signaling systems (1). Although various aspects of protein-phosphorylation systems have been investigated, uncertainties concerning the complex cellular responses in the second messenger system remain (2–4). Improved and sophisticated methods must be designed to estimate changes in the activities of cellular response elements after extracellular stimuli. While our comprehension of the biochemistry and molecular biology of protein kinases has progressed, the function of these enzymes in intact cells has been much more difficult to understand. For this reason researchers studying second messenger systems have long sought the development of specific and effective protein kinase inhibitors that would permit the definitive determination of the physiological role of the protein kinases (5). Protein kinase inhibitors can be used to determine the physiological significance of the protein phosphorylation systems in various types of cells. To elucidate the physiological function of each

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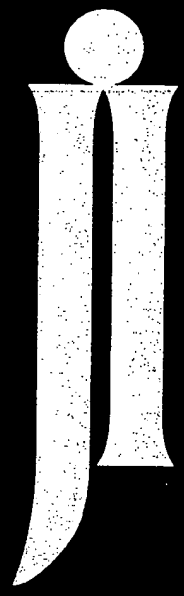

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Blocking of Heart Allograft Rejection by Intercellular Adhesion Molecule-1 Antisense Oligonucleotides Alone or in Combination with Other Immunosuppressive Modalities[1]

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Intercellular adhesion molecule-1 (ICAM-1) binds circulating leukocytes through interactions with β_2 integrins, LFA-1, and macrophage Ag-1. The phosphorothioate antisense oligodeoxynucleotide, IP-3082, specific for ICAM-1 mRNA inhibited ICAM-1, but not vascular cell adhesion molecule-1, mRNA induction and expression of ICAM-1 molecules by mouse endothelioma cells. Scrambled control oligonucleotides were ineffective. Untreated C3H (H-2^k) mice rejected C57BL/10 (H-2^b) heart allografts with a mean survival time of 7.7 ± 1.4 days. Administration i.v. of IP-3082 by a 7-day osmotic pump prolonged the survival of heart allografts in a dose-dependent fashion: 1.25 mg/kg, to 11 ± 0 days; 2.5 mg/kg, to 12 ± 2.7 days; 5 mg/kg, to 14.1 ± 2.7 days; and 10 mg/kg, to 15.3 ± 5.8 days (all $p < 0.01$). Control IP-1082 (10 mg/kg) was ineffective (7 ± 0.8 days). Although 7-day anti-LFA-1 mAb (50 μg/day; i.p.) prolonged allograft survival to 14.1 ± 2.7 days, the addition of IP-3082 (5.0 mg/kg × 7 days) induced donor-specific transplantation tolerance (>150 days). Furthermore, IP-3082 (5 mg/kg × 7 days) acted synergistically with antilymphocyte serum, rapamycin, and brequinar, but not cyclosporin A: a single antilymphocyte serum (0.2 ml) i.p. injection alone prolonged graft survival to 10 ± 0.5 days ($p < 0.01$) and in combination with IP-3082 (5 mg/kg), to 32.2 ± 8.3 days ($p < 0.001$); rapamycin (0.1 mg/kg × 7 days; i.v.) alone prolonged survival to 13 ± 7.5 days ($p < 0.01$), and with IP-3082, to 32.4 ± 8.9 days ($p < 0.03$); brequinar (0.5 mg/kg × 7 days; oral gavage) alone to 12 ± 2.4 days ($p < 0.05$), and with IP-3082 (5 mg/kg), to 38.8 ± 30.2 days ($p < 0.01$); in contrast, cyclosporin A (5 mg/kg × 7 days; i.v.) alone produced graft survival of 9.8 ± 1.3 days ($p < 0.1$ and in combination with IP-3082 (5 mg/kg), produced survival of 7.8 ± 3.5 days (NS). Thus, antisense oligonucleotides may proffer a selective gene-targeted immunosuppressive therapy for organ transplantation. *The Journal of Immunology,* 1994, 153: 5336.

Numerous studies have demonstrated the inhibition of viral and cellular gene expression after treatment of cells in culture with oligonucleotides designed to hybridize to a specific mRNA by Watson-Crick base pairing (1–3). Antisense oligonucleotides may inhibit protein expression by multiple mechanisms, including translational arrest, inhibition of RNA processing, and promotion of the degradation of the targeted RNA. Although unmodified phosphodiester oligonucleotides are effective in in vitro assays, their rapid hydrolysis by serum and cellular nucleases limits their utility in vivo (4, 5). In contrast, phosphorothioate oligodeoxynucleotides (PS-oligos),[3] in which sulfur is substituted for one of the nonbridging oxygen atoms in the phosphate backbone, demonstrate greatly increased stability in relation to serum and cellular nucleases and, therefore, may be very useful for in vivo applications (6).

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[3] Abbreviations used in this paper: PS-oligo, phosphorothiate oligonucleotides; ALS, antilymphocyte serum; BQR, brequinar; CsA, cyclosporin A; DOTMA/DOPE, *N*-[1(-2,3[-dioleyloxy)propyl]]-*N*,*N*,*N*-trimethylammonium chloride/dioleoylphosphatidylethanolamine; ICAM-1, intercellular adhesion molecule-1; MAC-1, macrophage Ag-1; RAPA, rapamycin; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late Ag-4; MST, mean survival time; CI, combination index; q.o.d., every second day; GTPDH, glyceraldehyde triphosphate dehydrogenase.

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As was recently demonstrated, PS-oligos designed to hybridize to different regions of human intercellular adhesion molecule-1 (ICAM-1; CD54) mRNA inhibited ICAM-1 expression in a sequence-specific manner (7). The most active PS-oligo hybridized to sequences located in the 3′-untranslated region of ICAM-1 mRNA. The ICAM-1 molecule belongs to the Ig-related cell adhesion molecule family and is expressed at a low level on the surface of endothelial cells and leukocytes (8–10). In response to pro-inflammatory cytokines, such as IL-1, TNF, and IFN-γ, the expression of ICAM-1 molecules is markedly increased on a variety of cells, including endothelial cells and fibroblasts, and is induced on keratinocytes, smooth muscle cells, and cardiac myocytes (11). ICAM-1-expressing endothelial cells bind circulating leukocytes through interactions with the β_2 integrins, namely LFA-1 (CD11a) and MAC-1, thereby facilitating emigration of leukocytes out of the vasculature (12, 13).

Furthermore, ICAM-1 interaction with LFA-1 has been shown to enhance T cell interaction with APC, T cell-dependent activation of B cells, and the killing of target cells by neutrophils, NK cells, or Ag-specific cytotoxic T cells (14–18). Adhesion of leukocytes to endothelial cells requires three overlapping steps, namely rolling, chemoattraction, and strong adhesion (19, 20). Upon activation by pro-inflammatory mediators, a family of selectins, namely granule-external membrane protein (P-selectin; CD62P), endothelial-leukocyte adhesion molecule (E-selectin; CD62E), and lymph node homing receptor (L-selectin; CD62L) bind to the carbohydrate ligands (sialylated Lewis^a and Lewis^x), thereby triggering an initial tethering of flowing leukocytes to the vessel wall (10, 21). The rolling of leukocytes along the surface of the endothelial cells enhances their exposure to chemoattractants that transduce signals, thereby increasing the avidity of integrins (22). The firm attachment of leukocytes to endothelial cells involves an interaction between integrins (LFA-1, MAC-1, and very late Ag-4 (VLA-4) molecules) on circulating leukocytes and Ig-related cell adhesion molecules (ICAM-1, ICAM-2, ICAM-3, and vascular cell adhesion molecule-1 (VCAM-1)) on the endothelial cells (23). Increased expression of ICAM-1 molecules correlates with increased leukocyte infiltration followed by the rejection of organ allografts in both humans and mice (24–26). Direct evidence for the involvement of ICAM-1 and LFA-1 molecules in allograft rejection was provided by the treatment of heart allograft recipients with anti-ICAM-1 (YN1/1.7.4) and anti-LFA-1 (KBA) mAb (27). A 7-day i.p. therapy with anti-ICAM-1 or anti-LFA-1 mAb prolonged the survival of heart allografts. However, the combination of anti-ICAM-1 and anti-LFA-1 mAb induced donor-specific transplantation tolerance. These findings suggest that reduction in the expression of ICAM-1 followed by decreased appearance of LFA-1-expressing cells may produce potent in vivo immunosuppressive activity. We found that mouse ICAM-1 antisense PS-oligos inhibited the rejection of heterotopic cardiac allografts and, in combination with anti-LFA-1 mAb, induced donor-specific transplantation tolerance. Furthermore, ICAM-1 antisense PS-oligos combined with antilymphocyte serum (ALS), rapamycin (RAPA), or brequinar (BQR), but not with cyclosporin A (CsA), synergistically inhibited the rejection of heart allografts.

Materials and Methods

Cells and reagents

Fetal bovine serum was purchased from HyClone (Logan, UT). DMEM and Dulbecco's PBS were purchased from Irvine Scientific (Irvine, CA). Opti-MEM serum-free medium was obtained from Life Technologies (Grand Island, NY). Primaria 96-well plates were obtained from Falcon Labware (Lincoln Park, NJ). Human rTNF-α and mouse IFN-γ were purchased from R&D systems (Minneapolis, MN). Anti-LFA-1 mAb were obtained from Dr. Yogita (Juntendo University School of Medicine, Tokyo, Japan), and anti-VCAM-1 mAb were purchased from Genzyme (Cambridge, MA). Anti-ICAM-1 mAb (YN1/1.7.4; American Type Culture Collection, Rockville, MD) were purified from ascites on a protein G column. DOTMA/DOPE (*N*-[1(-2,3-dioleyloxy)propyl]]-*N,N,N*-trimethyl-ammonium chloride/dioleoyl phosphatidylethanolamine) solution (lipofectin), biotinylated goat anti-mouse IgG, and β-galactosidase-conjugated streptavidin were purchased from Bethesda Research Labs (Bethesda, MD). The DNA synthesizer reagents, controlled pore glass-bound and β-cyanoethyldiisopropylphosphoramidites, were obtained from Applied Biosystems (Foster City, CA). Centrex filters were purchased from Schleicher & Schuell (Keene, NH). Zeta-Probe nylon blotting membranes were purchased from Bio-Rad (Richmond, CA). QuickHyb solution was purchased from Strategene (La Jolla, CA), and the cDNA labeling kit, Prime-a-Gene, was obtained from Promega (Madison, WI).

PS-oligo synthesis

PS-oligos were synthesized on a 0.5-mmole scale on a Milligen model 8800 DNA synthesizer by using modified phosphoramidite chemistries with β-cyanoethoxyphosphoramidites (7). A crude product of approximately 70% purity was further purified by orthagonal column chromatography by using a Millipore HC18-HA column followed by anion exchange chromatography on a Millipore Q-15 strong anion exchanger. The purified material was ethanol precipitated, redissolved, and further desalted by ultrafiltration. The samples were depyrogenated by ultrafiltration with endotoxin levels that had been reduced to below detectable levels. The sequences of the PS-oligos were: IP-3068, AGCTGCGCTGCTACCTGCAC; IP-3069, GCCCATTGCAGGGCCAGGGC; IP-3066, GGGTTGAAGCC ATTGCAGGG; IP-3070, CTCATCCAGCAGGCTCAGGG; IP-3065, CCAGAGGAAGTGGCTGAGGG, IP-3082, TGCATCCCCCAGGCCAC CAT; IP-3806, CAAGTGTGCATCCCCCAGGC; IP-3083, TTGGGAC AATGTCTCAGCTT, IP-3084, TGCCAGTCCACATAGTGTTT; and IP-3099, TGCTTACCCTCCCACAGCAG. The control PS-oligo sequences were: IP-3823, TGCCCCTCACCAGCACGCAT, IP-8997, TCGCATC GACCCGCCCACTA, and IP-4189, CAGCCATGGTTCCCCCCAAC, which were scrambled IP3082 sequences; and IP-1082, GCCGAGGTC CATGTCGTACGC, which targets the herpes virus UL-13 gene sequence.

PS-oligo treatment

The bEND.3 cells were kindly provided by Dr. Werner Risau, Max-Planck Institute, Planegg-Martinsreid, Germany. Cells were treated with indicated concentration of PS-oligo in the presence of 15 μg/ml DOTMA/DOPE liposome formulation for 4 h. ICAM-1 expression was induced by treatment with 5 ng/ml human rTNF-α and 1000 U/ml murine IFN-γ for 16 h (7). Cells were fixed with 95% ethanol, and ICAM-1 expression was quantified by incubation with ICAM-1 mAb (YN1/1.7.4), followed by incubation with a biotinylated goat anti-rat IgG Ab and streptavidin conjugated β-galactosidase. Results are expressed as the percentage of control ICAM-1 expression, which was calculated as follows: [((ICAM-1 expression for oligonucleotide-treated cytokine-induced cells) − (basal ICAM-1 expression))/((cytokine-induced ICAM-1 expression) − (basal ICAM-1 expression))] × 100. Both basal and cytokine-treated cells were pretreated with DOTMA.

RNA isolation and analysis

Total cellular RNA was isolated by cellular lysis in 4 M guanidinium isothiocynate followed by a cesium chloride gradient (28). Total RNA was separated on a 1% agarose gel containing 1.1% formaldehyde and transferred to nylon membranes. Blots were hybridized for 1 to 2 h in QuickHyb solution with the ICAM-1 cDNA probe, which had been labeled with [^{32}P]dCTP with use of random oligonucleotide primers. Blots were washed with 2X SSC containing 0.1% SDS at 25°C then washed in 0.1X SSC containing 0.1% SDS at 60°C. Quantitation of RNA expression was performed by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Mice

C3H (H-2^k), C57BL/10 (H-2^b), C57BL/6 (H-2^b), and BALB/c (H-2^d) female mice, 4 to 6 wk old, were purchased from Harlan Sprague-Dawley (Indianapolis, IN), housed in cages in room with controlled light/dark cycles, and supplied food and water ad libitum. All experiments were approved by the Animal Welfare Committee and conducted in accordance with the University of Texas Guidelines.

Heart transplantation

Heterotopic heart transplantation was performed according to the previously described method (29). Donor hearts were perfused through the vena cava and aorta with cold heparinized saline before harvesting and ligation of the vena cava and pulmonary veins. The donor pulmonary artery was anastomosed to the vena cava of the recipient, and the donor aorta was anastomosed to the recipient aorta, by using 11-0 nylon suture (Ethicom Inc., Sommerville, NY). Cold ischemia time was less than 30 min. Heart allograft function was examined by daily palpation, and the day of heartbeat cessation was regarded as the day of rejection.

Immunosuppressive modalities

CsA (Sandoz, Basel, Switzerland) dissolved in cremophor (Sigma Chemical Co., St. Louis, MO) was delivered via jugular venous infusion by a 7-day osmotic pump (Alzet, Palo Alto, CA). RAPA (Wyeth Ayerst, Rouses Point, NY) in a dilutant (Tween 80, 10%; *N-N*-dimethylacetamide, 20%; and polyethylene glycol 400, 70%) was infused i.v. by 7-day osmotic pump. BQR (DuPont, Wilmington, DE) diluted in distilled water was administered every second day (q.o.d.) by oral gavage for 7 days. Rabbit anti-mouse ALS (Accurate Chemicals, New York, NY) was injected once i.p. two days before grafting.

Statistical analysis

Heart allograft survivals are presented as a mean survival time (MST ± SD), with comparison among groups being performed by Gehan's survival test. The median-effect principle (30, 31) is on the basis of the premise that the effect of each agent is related to its dose and, therefore, may be calculated by using the following equation:

$$\left(\frac{fa}{fu}\right) = \left(\frac{D}{Dm}\right)^m$$

Where fa and fu represent the fractions of the system that are affected (percent inhibition or days of survival beyond controls) and unaffected (1-fa), respectively, by the drug at dose D. Full protection (fa = 1) is defined as at least a 50-day survival of heart allografts. *Dm* is the dose required for 50% inhibition (ED_{50}), the median effect; *m* is a coefficient that describes the sigmoidicity of the dose-effect curve. Logarithmic conversion of the median-effect equation linearizes the relationship:

$$\log\left(\frac{fa}{fu}\right) = m \log (D) - m \log (Dm)$$

This relation defines *m* as the slope of the plot log (fa/fu) vs log dose and log *Dm* as the X-intercept when log (fa/fu) = 0 or fa = fu = 0.5. The *m* value describes the shape of the dose-effect curve, which is hyperbolic when *m* equals 1 and sigmoidal or negatively sigmoidal when *m* is higher or lower than 1, respectively. The linear regression coefficient *r* describes the "goodness" of the fit of the data to the median-effect principle. A minimum, *r* of 0.75 was required to conclude that the data obey the median-effect principle. The interaction between two drugs was assessed by using the combination index (CI) method (31) for the doses to achieve *x*% inhibition (days of graft survival):

$$CIX = \frac{D_1 \text{ combined}}{(Dx)_1 \text{ alone}} + \frac{D_2 \text{ combined}}{(Dx)_2 \text{ alone}}$$

for the mutually exclusive case, where both drugs have the same or similar modes of action, or the more conservative expression:

$$CIX = \frac{D_1 \text{ combined}}{(Dx)_1 \text{ alone}} + \frac{D_1 \text{ combined}}{(Dx)_2 \text{ alone}} + \frac{(D_1 \text{ combined})\,(D_2 \text{ combined})}{[(Dx)_1 \text{ alone}]\,[(Dx)_2 \text{ alone}]}$$

for the mutually nonexclusive case, where each drug has a different mode of action. Computer software (32) was used to automatically determine the dose-effect parameters (*Dm*, *m*, and *r*) and the CI values.

Results

Selection of ICAM-1 antisense PS-oligos by in vitro analysis

Comparison of the nucleotide sequences of human and murine ICAM-1 cDNAs revealed that the cDNA sequences are conserved only 65% (7, 33). The degree of conservation was even lower within the untranslated 3′-regions, where the most active human antisense PS-oligos were identified. Therefore, it was necessary to identify PS-oligos that selectively target murine ICAM-1 mRNA. To select the most effective PS-oligos for in vivo studies, ten PS-oligos (20 mer) were designed to hybridize to different regions on the mouse ICAM-1 mRNA (Fig. 1*A*). In particular, we selected three mouse sequences in the untranslated 5′-region and seven mouse sequences in the untranslated 3′-region. The PS-oligos were screened by an ELISA in the presence of a cationic liposome formulation (DOTMA/DOPE) for inhibition of ICAM-1 induction in a murine endothelioma cell line, bEND.3. The bEND.3 cells expressed a basal level of ICAM-1 that increased significantly after treatment with a combination of human TNF-α and murine INF-γ. A 4-h preincubation with the PS-oligo, followed by a 16-h culture with cytokines, produced different effects on ICAM-1 expression. Although all of the ICAM-1 antisense PS-oligos inhibited cytokine-induced ICAM-1 expression (Fig. 1*B*), IP-3082 and IP-3806, which hybridized to the 3′-untranslated region of the ICAM-1 mRNA, were able to lower ICAM-1 protein expression to below the basal level of expression (Fig. 1*B*). IP-3082 was chosen for further evaluation by using an in vitro cellular assay to measure ICAM-1 and VCAM-1 mRNA expression (Fig. 2). Treatment of bEND.3 cells with 50 nM IP-3082 selectively reduced cytokine-induced ICAM-1 mRNA by 90% (Fig. 2*A*). The effect of IP-3082 on ICAM-1 mRNA was sequence specific; three scrambled (IP-3823, IP-8997, and IP-4189) and one unrelated (IP-1082) PS-oligos failed to affect ICAM-1 mRNA expression. In fact, two scrambled PS-oligos, IP-8997 and IP-4189, slightly enhanced ICAM-1 mRNA expression. The effect on ICAM-1 was selective: IP-3082 and two controls (IP-3823 and IP-8997) only slightly reduced

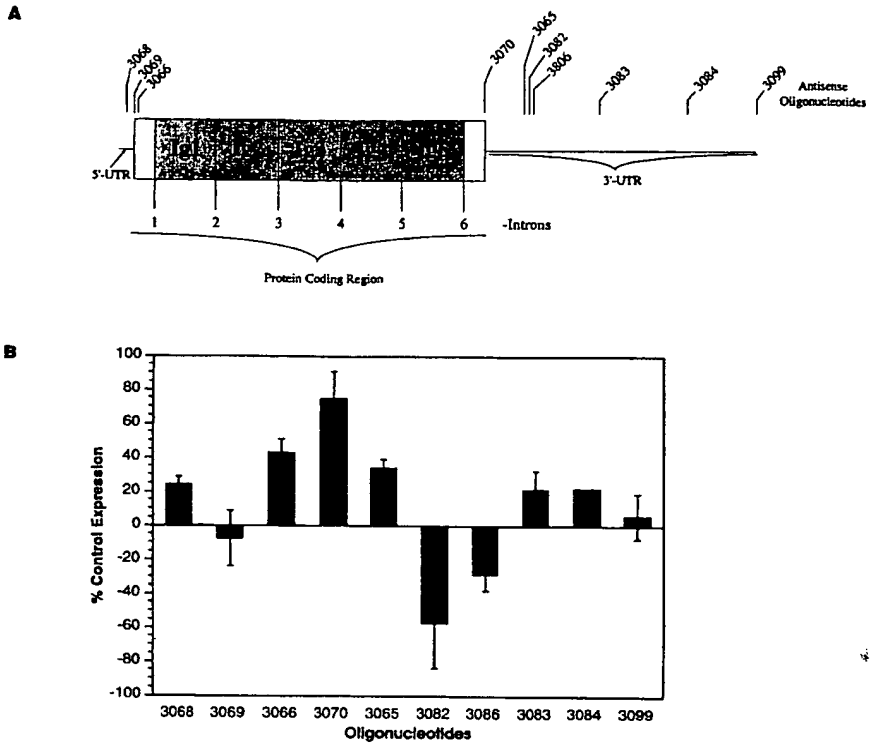

FIGURE 1. Specific inhibition by I-3082 of ICAM-1 mRNA expression. bEND.3 cells treated for 4 h with 50 nM ICAM-1 antisense I-3082, three scrambled control PS-oligos, I-3823, I-8997, and I-4189, or unrelated I-1082 controls were stimulated for 2 h with 5 ng/ml TNF-α and 1000 U/ml murine IFN-γ. Total cellular RNA was analyzed by Northern blot using a random primed murine ICAM-1 (*A*), VCAM-1 (*B*), or G3PDH (*C*) cDNA probe. The ICAM-1, VCAM-1, or G3PDH mRNA was quantified by a PhosphorImager (Sunnyvale, CA). The G3PDH probe demonstrated equal loading of the gel lanes.

VCAM-1 mRNA expression (up to 25%). Furthermore, IP-3082 and control PS-oligos did not affect the glyceraldehyde triphosphate dehydrogenase (G3PDH) transcripts (Fig. 2*C*). These results showed that, among 10 ICAM-1 antisense PS-oligos, IP-3082, which hybridizes to the 3′-end untranslated region, effectively inhibited ICAM-1 protein expression and reduced the level of ICAM-1 mRNA in bEND.3 endothelial cells. Thus, in vitro data strongly suggest that IP-3082 inhibits ICAM-1 expression by means of an antisense mechanism.

Effect of ICAM-1 antisense PS-3082 on heart allograft survival

The in vivo activity of PS-oligos was examined in a mouse heterotopic cardiac transplant model (29). ICAM-1 antisense PS-oligo, IP-3082, unrelated IP-1082 control, or scrambled IP-4189 control was delivered i.v. by a 7-day osmotic pump. Untreated C3H(H-2^k) mice rejected C57BL/10 (H-2^b) vascularized heart allografts at a MST of 7.7 ± 1.4 days (Table I). A 7-day infusion of the control IP-1082 at a dose of either 5 or 10 mg/kg/day did not affect allograft survival. Similarly, a scrambled IP-4189 control (10 mg/kg) did not significantly affect heart allograft survival (9 ± 0.8 days; NS). In contrast, infusion of the ICAM-1 antisense IP-3082 prolonged allograft survival in a dose-dependent fashion: 1.25 mg/kg/day of IP-3082 prolonged graft survival to 11 ± 0 days; 2.5 mg/kg/day, to 12 ± 2.7 days; 5 mg/kg/day, to 14.1 ± 2.7 days; and 10 mg/kg/day, to 15.3 ± 5.8 days (all $p < 0.01$). Extended 14-day treatment with IP-3082 (5 mg/kg/day) further increased graft survival up to 30 days. Almost identical results were produced in two additional donor-recipient combinations, namely BALB/c (H-2^d) to C3H and C57BL/6 (H-2^b) to BALB/c (data not shown). The effectiveness of the immunosuppression was documented by histologic examination of grafts on day 6 after transplantation. Syngeneic C57BL/10 hearts transplanted to C57BL/10 recipients showed mild infiltration with mononuclear cells (10% of the myocardium) compared with normal controls (Fig. 3, *A* and *B*). Heart allografts from untreated recipients displayed strong infiltration with

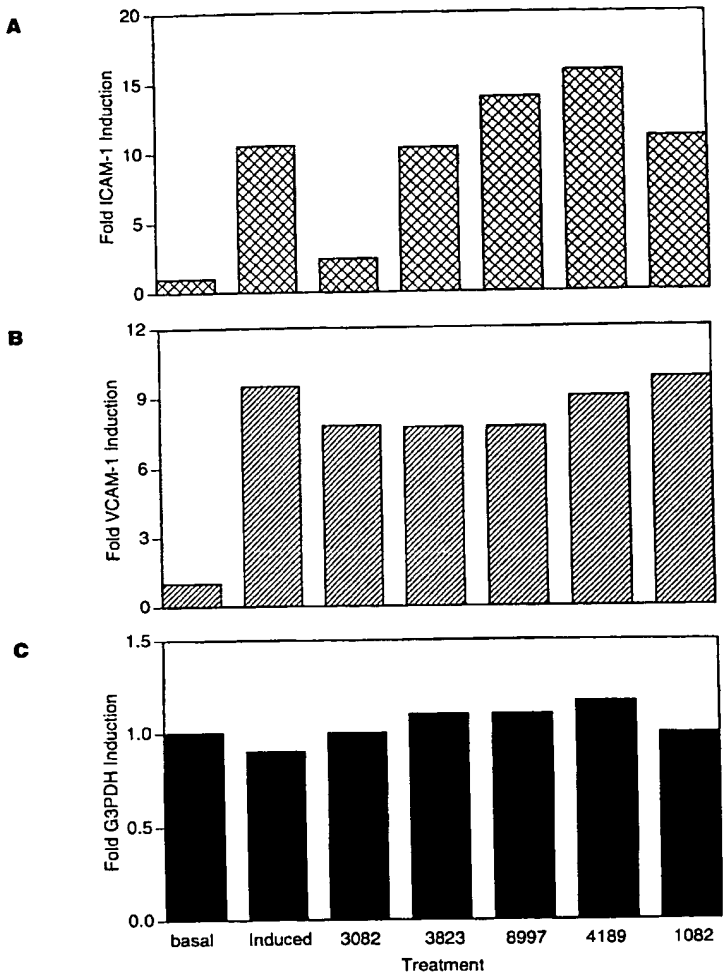

FIGURE 2. Histology of C57BL/10 hearts. *A*) normal, control heart. *B*) syngeneic heart graft 6 days after transplantation demonstrating mild infiltration of 10% of the tissue. *C*) allogeneic heart graft 6 days after grafting to untreated recipient demonstrating massive infiltration of leukocytes and neutrophils with severe necrosis and interstitial hemorrhage. *D*) allogeneic graft 6 days after transplantation to recipient treated with 10 mg/kg/day ICAM-1 I-3082 demonstrating mild infiltration of 20% of the tissue. All sections were fixed in 10% formalin and stained with H&E. Original magnification is ×200.

Table I. *Effect of ICAM-1 antisense IP-3082 on C57BL/10 (H-2^b) heart allograft survival in C3H (H-2^b) recipients*[a]

Treatment				
PS-oligos (days)	mg/kg/day	Graft Survival (days)	MST ± SD	*p*
None	–	6, 7 × 3, 8, 9, 10	7.7 ± 1.4	–
IP-1082 (×7)	5.0	6, 7 × 3, 8 × 2	7.1 ± 0.7	NS
	10.0	6, 7 × 2, 8	7.0 ± 0.8	NS
IP-4189 (×7)	10.0	8, 9 × 2, 10	9.0 ± 0.8	NS
IP-3082 (×7)	1.25	11 × 3	11.0 ± 0	0.001
	2.5	9, 10, 12, 13, 16	12.0 ± 2.7	0.01
	5.0	10, 12 × 2, 13, 16 × 2, 17 × 2	14.1 ± 2.7	0.01
	10.0	12 × 2, 13, 24	15.3 ± 5.8	0.01
IP-3082 (×14)	5.0	16, 17, 29, 30	23.0 ± 7.5	0.001

[a] Recipients were untreated or infused i.v. by 7-day (×7) osmotic pump with 5 or 10 mg/kg control IP-1082 (17), 10 mg/kg control IP-4189, or 1.25, 2.5, 5, or 10 mg/kg antisense IP-3082 starting at the day of transplantation. In one group of mice, 5 mg/kg IP-3082 was infused i.v. for 14 (×14) days. Heart allografts were evaluated daily by palpation, and the day of heart beat cessation was considered the day of rejection.

mononuclear cells and neutrophils. This effect was associated with severe necrosis and mineralization, which formed a dense band that affected 60% of the epicardium, myocardium, and papillary muscles (Fig. 3*C*). In contrast, heart allografts from recipients treated with IP-3082 (5 mg/kg/day) showed only scattered infiltration with mononuclear

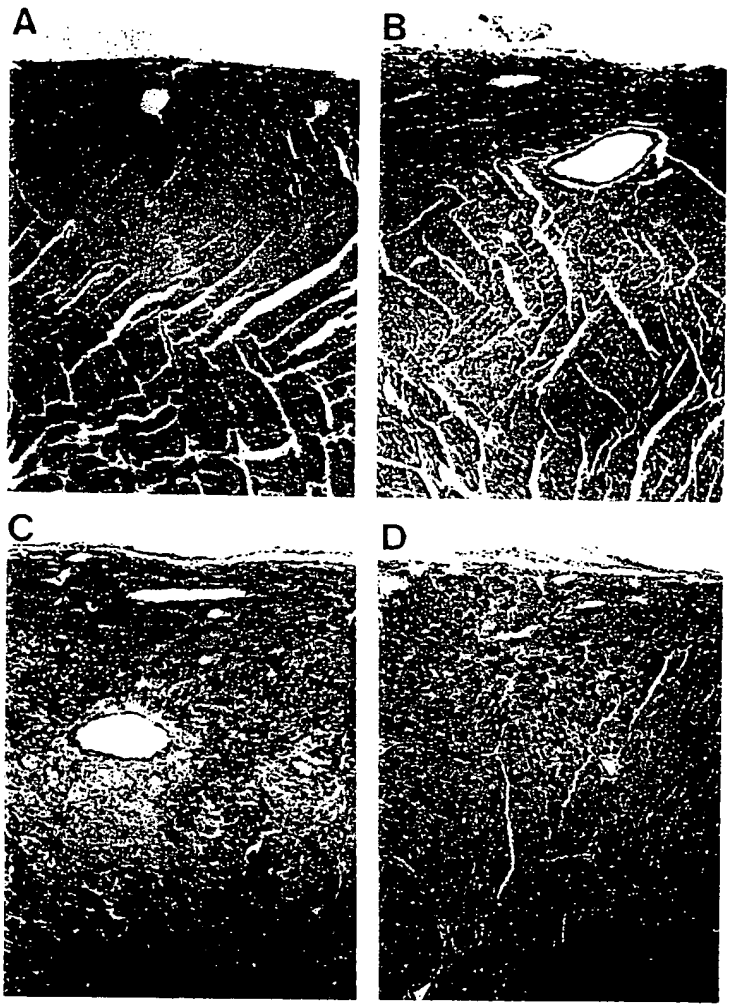

FIGURE 3. Combined effect of ICAM-1 antisense I-3082 and anti-LFA-1 mAb (KBA) on heart allograft survival. C3H recipients of C57BL/10 hearts were either untreated or treated with daily i.p. injection for 7 days of KBA Ab (50 μg/day) alone, I-3082 (5 mg/kg/day) i.v. by 7-day osmotic pump alone, or a combination of KBA mAb (50 μg/day) i.p. and I-3082 (5 mg/kg/day) i.v. for 7 days.

cells in 20% of the myocardium (Fig. 3*D*). Thus, we conclude that ICAM-1 antisense IP-3082 inhibits infiltration and subsequent destruction of heart allograft tissue by host cells.

Combined effect of ICAM-1 antisense IP-3082 and anti-LFA-1 mAb on heart allograft survival

The in vivo effects of the ICAM-1 antisense PS-oligo IP-3082 in conjunction with anti-LFA-1 mAb (KBA) were examined in the C57BL/10 to C3H combination. A 7-day treatment with i.p. injections of anti-LFA-1 mAb (50 μg/day) or i.v. infusion of IP-3082 (5 mg/kg/day) prolonged allograft survival to 10.6 ± 4.6 days and 14.1 ± 2.7 days, respectively (Fig. 4). Combined treatment with the two agents for 7 days resulted in indefinite survival of heart allografts (>150 days; $p < 0.001$). The interaction of IP-3082 and anti-LFA-1 mAb was evaluated by the median effect analysis (30). The MST values revealed a dose-dependent effect of individual agents: linear regression coefficient (r) values were higher than 0.75, thereby allowing further analysis. The r value for IP-3082 was calculated on the basis of the results presented in Table I. Three individual doses of anti-LFA mAb were examined: 25 μg/day for 7 days was ineffective (7.7 ± 0.6); 50 μg/day prolonged graft survival to 10.6 ± 4.6 days; and 100 μg/day prolonged survival to 15.0 ± 5.9 days ($p < 0.01$). CI value calculated for a combination of 5 mg/kg/day IP-3082 and 50 μg/day anti-LFA-1 mAb was 0.001, indicating strong synergism (CI = 1 shows additive, and CI < 1 or CI > 1 shows synergistic or antagonistic interaction, respectively). Recipients bearing C57BL/10 hearts for 65 days ($n = 4$) were transplanted with donor-type C57BL/10 and third-party BALB/c (H-2^d) skin allografts. The induction of transplantation tolerance was confirmed by permanent acceptance of donor-type skin grafts (>100 days) and acute rejection of third party grafts in 9 ± 0 days (data not shown). Control C3H mice ($n = 5$) rejected C57BL/10 and

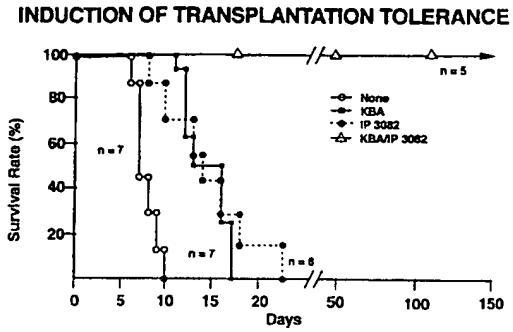

FIGURE 4. Identification of a murine ICAM-1 antisense PS-oligo. *A*) the approximate position of 10 PS-oligos designed to hybridize to different regions of the ICAM-1 mRNA: I-3068, I-3069, and I-3066 in the untranslated 5'-end region; and I-3083 and I-3084 in the untranslated 3'-end regions. *B*) murine endothelioma cells, bEND.3, were treated with 300 nM of the indicated PS-oligo in the presence of 15 μg/ml DOTMA/DOPE liposome formulation for 4 h in serum-free medium, as previously described by Chiang et al. (7) and Bennett et al. (57). Medium was replaced with DMEM containing 10% fetal bovine serum, and ICAM-1 expression was induced with human TNF-α (5 ng/ml) and murine IFN-γ (1000 U/ml). ICAM-1 expression was quantified 16 h after induction by an ELISA (7). Under basal conditions, approximately 35% of the bEND.3 cells express ICAM-1 as determined by flow cytometry (data not shown).

BALB/c grafts in 9.2 ± 0.8 days and 8.1 ± 0.6 days, respectively. These results indicate that the combination of ICAM-1 antisense PS-oligo, PS-3082, and anti-LFA-1 mAb induces donor-specific transplantation tolerance.

Effect of ICAM-1 antisense IP-3082 in combination with ALS, RAPA, BQR, or CsA on heart allograft survival

The interaction of IP-3082 with different immunosuppressive agents, namely ALS (34), RAPA (35, 36), BQR (37), and CsA (38–40), was examined in C3H recipients of C57BL/10 heart allografts. Median effect analysis was used to calculate the individual effects of IP-3082 (Table I), ALS (Table II), RAPA (Table III), BQR (Table IV), and CsA (Table V) as well as the combined effects of IP-3082 with ALS, RAPA, BQR, or CsA. A single i.p. injection of ALS two days before transplantation prolonged graft survival: 0.1 ml prolonged graft survival to 9 ± 0 days; 0.2 ml, to 10.4 ± 0.5 days; and 0.4 ml, to 14 ± 2.1 days (all $p < 0.01$; Table II). The combination of 0.2 ml ALS and IP-3082 (5, 10, or 20 mg/kg/day × 7 days) extended allograft survivals to 32.2 ± 8.3 days, 37.0 ± 5.8 days, and 72.0 ± 49.1 days, respectively (all $p < 0.01$; CI < 0.001). RAPA (0.05, 0.1, or 0.2 mg/kg/day) alone delivered i.v. by a 7-day osmotic pump immunosuppressed rejection in a dose-dependent fashion (Table III). Combining 0.1 mg/kg RAPA with 5 mg/kg IP-3082 prolonged graft survival to 32.4 ± 8.9 days, and combining it with 10.0 mg/kg IP-3082, to 36.3 ± 6.1 days (both $p <$ 0.01; CI < 0.02). Oral gavage with BQR (0.5, 1, and 2 mg/kg/day) delivered every second day (q.o.d.) for 7 days prolonged allograft survival to 12 ± 2.4 days, 17.6 ± 3.4 days, and 20 ± 4.1 days, respectively (Table IV; all <0.01); the combination of 0.5 mg/kg BQR and 5 mg/kg IP-3082 resulted in a MST of 38.8 ± 30.2 days ($p < 0.01$; CI = 0.007). Although a 7-day i.v. infusion of 2.5 or 5 mg/kg/day CsA was ineffective, 10 or 20 mg/kg/day CsA prolonged allograft survival. Adding IP-3082 (5 or 10 mg/kg/day) to 5 mg/kg/day CsA did not improve graft survivals with CI values of 14.1 and 51, respectively (Table V). Similarly, combining control IP-1082 (5 mg/kg) and CsA (5 mg/kg) did not affect heart allograft survival (9.2 ± 2.2 days; NS; data not shown). These results show that ICAM-1 antisense IP-3082 synergistically interacts with different immunosuppressive modalities (ALS, RAPA, and BQR), but not with CsA, to block allograft rejection.

Discussion

These experiments were designed to select an ICAM-1 antisense PS-oligo for gene-targeted immunosuppressive therapy to block heart allograft rejection. The results documented that in vitro screening allowed the determination of a very effective target region for PS-oligo on mouse ICAM-1 mRNA. Interestingly, the most active PS-oligo (IP-3082) hybridized to the 3'-untranslated region of ICAM-1 mRNA. IP-3082 inhibited in vitro expression of cytokine-induced ICAM-1 protein on bEND.3 endothelioma cells. Furthermore, under the same experimental conditions, IP-3082 inhibited cytokine-induced ICAM-1 mRNA by 95%. Similar results were observed during analysis of 35 PS-oligos that target human ICAM-1 mRNA, which were the most effective PS-oligos hybridized to the 3'-untranslated region (7, 41). The human ICAM-1 antisense PS-oligos seem to inhibit ICAM-1 expression by a mechanism involving RNase H hydrolysis of the target mRNA. The inhibitory activity was lost when the ICAM-1 antisense PS-oligos were modified by substitution on the 2'-position of the sugar by fluoro, *O*-methyl, or *O*-propyl (7, 41). Such substitutions enhanced binding of the PS-oligo to the target mRNA, but do not support RNase H hydrolysis. Similar results were obtained with mouse antisense PS-oligos (data not shown), which suggests that PS-3082 inhibits ICAM-1 expression, at least in part, through a RNase H mechanism.

The in vitro studies were extended to an in vivo analysis to examine the effect of the ICAM-1 antisense PS-oligo, IP-3082, in a model of complex inflammatory response to organ allografts. Previously, it has been demonstrated that anti-ICAM-1 mAb significantly prolongs heart allograft survival, implicating ICAM-1 in the rejection process (27). The ICAM-1 antisense PS-oligo, IP-3082, extended heart allograft survival in a dose-dependent fashion when

Table II. *Combined effect of ALS and ICAM-1 antisense IP-3082 on heart allograft survival*[a]

Treatment					
IP-3082 ALS (ml)	(mg/kg/day)	Heart Survival (days)	MST ± SD	*p*	CI
None	–	6, 7 × 3, 8, 9, 10	7.7 ± 1.4	–	–
0.1	–	9 × 4	9.0 ± 0.0	0.01	–
0.2	–	10 × 3, 11 × 2	10.4 ± 0.5	0.01	–
0.4	–	11, 14, 15, 16	14.0 ± 2.1	0.01	–
0.2	5.0	20, 30, 31, 39, 41	32.2 ± 8.3	0.01	0.003
0.2	10.0	32 × 2, 41, 43	37.0 ± 5.8	0.01	0.001
0.2	20.0	33, 34, 54, 89, >150	72.0 ± 49.1	0.01	0.001

[a] ALS was injected i.p. one time 2 days before transplantation; IP-3082, i.v. by a 7-day osmotic pump.

Table III. *Combined effect of RAPA and ICAM-1 antisense IP-3082 on heart allograft survival*[a]

Treatment					
RAPA (mg/kg/day)	IP-3082 (mg/kg/day)	Heart Survival (days)	MST ± SD	*p*	CI
None	–	6, 7 × 3, 8, 9, 10	7.7 ± 1.4	–	–
0.05	–	6 × 2, 7, 9 × 2	7.4 ± 1.4	NS	–
0.1	–	10, 11, 20 × 2, 21	13.0 ± 7.5	0.01	–
0.2	–	12, 14, 17, 18, 39	20.0 ± 10.9	0.01	–
0.1	5.0	23, 24, 33, 39, 43	32.4 ± 8.9	0.01	0.03
0.1	10.0	32 × 2, 36, 45	36.3 ± 6.1	0.01	0.02

[a] RAPA was delivered i.v. by a 7-day osmotic pump; IP-3082, as in Table II.

Table IV. *Combined effect of BQR and ICAM-1 antisense IP-3082 on heart allograft survival*[a]

Treatment					
BQR (mg/kg/day)	IP-3082 (mg/kg/day)	Heart Survival (days)	MST ± SD	*p*	CI
None	–	6, 7 × 3, 8, 9, 10	7.7 ± 1.4	–	–
0.5	–	9, 11 × 2, 14, 15	12.0 ± 2.4	0.05	–
1.0	–	13, 16, 18, 19, 22	17.6 ± 3.4	0.01	–
2.0	–	15, 17, 20, 23, 25	20.0 ± 4.1	0.01	–
0.5	5.0	21, 24, 28, 29, 31, >100	38.8 ± 30.2	0.01	0.001

[a] BQR was delivered every second day by oral gavage; IP-3082 was delivered as in Table II.

administered for 7 days i.v. by osmotic pumps. Treatment of C3H recipients with 5 mg/kg/day IP-3082 for 7 days prolonged C57BL/10 heart allograft survival from 7.7 ± 1.4 days in untreated controls to 14.1 ± 2.7 days, producing an effective immunosuppression 5 to 7 days beyond termination of therapy. Similar results were obtained by using the 7-day anti-ICAM-1 mAb (27). Thus, the ICAM-1 antisense PS-oligo and anti-ICAM-1 mAb produced almost identical in vivo immunosuppressive effects.

Although these results are consistent with the antisense mechanism of immunosuppression, we cannot definitively conclude that IP-3082 acts by such a mechanism in vivo. The in vitro results suggest that IP-3082 acts in a sequence-specific fashion, that is, by blocking ICAM-1, but not VCAM-1 or G3PDH, expression. In the in vivo heart allograft model, the same IP-3082 prolonged heart allograft survival. A similar effect was not observed after treatment with a nonspecific IP-1082 or a scrambled IP-4189 control. Thus, both the in vitro and in vivo IP-3082 effects are sequence specific and, therefore, are consistent with antisense mechanisms. The IP-3082 therapy reduced leukocyte infiltration of heart allografts, as would be expected by the activity of an ICAM-1 antisense PS-oligo. To inhibit ICAM-1 expression, the IP-3082 may act directly on ICAM-1 molecules present on the allograft or indirectly on the inflammatory cells that migrate to the graft. The latter effect may result in a decreased production of cytokines, leading to diminished ICAM-1 expression and reduced infiltration of heart allografts. Experiments are in progress to elucidate the in vivo mechanism of immunosuppression by IP-3082 antisense PS-oligo in this complex transplant model. Furthermore, IP-3082 has shown activity in other inflammatory models, such as dextran sulfate-induced colitis and collagen-induced arthritis, thereby demonstrating that the same PS-oligo

Table V. *Combined effect of CsA and ICAM-1 antisense IP-3082 on heart allograft survival*[a]

Treatment					
CsA (mg/kg/day)	IP-3082 (mg/kg/day)	Heart Survival (days)	MST ± SD	*p*	CI
None	–	6, 7 × 3, 8, 9, 10	7.7 ± 1.4	–	–
2.5	–	6 × 2, 7, 8, 9	7.2 ± 1.3	NS	–
5.0	–	8, 9, 10, 11 × 2	9.8 ± 1.3	NS	–
10.0	–	8, 13, 14, 19, 21	15.0 ± 5.1	0.01	–
20.0	–	14 × 3, 17 × 2	15.2 ± 1.6	0.01	–
5.0	5.0	7, 8, 9 × 2, 10 × 2	8.8 ± 1.1	NS	14.1
10.0	5.0	6 × 3, 7, 14	7.8 ± 3.5	NS	51.0

[a] CsA was delivered by a 7-day osmotic pump; I-3082 was delivered as in Table II.

activity in vivo is not limited to a single model (C. F. Bennett and T. Geiger, unpublished observations).

The antisense ICAM-1 PS-oligo, IP-3082, interacted synergistically with other immunosuppressive agents, but not with CsA, to block heart allograft rejection. Most strikingly, the combination of IP-3082 and anti-LFA-1 mAb produced donor-specific transplantation tolerance. Similar results were described for the combination of anti-ICAM-1 and anti-LFA-1 mAb (27). Thus, both experimental procedures support the conclusion that ICAM-1 and LFA-1 are important for allograft rejection. ICAM-1 are expressed at low levels on normal endothelial cells, but not on myocytes or endocardium (42). During allograft rejection, ICAM-1 expression increases on endothelial cells and is induced on myocytes and the endocardium (43). In contrast, β_2 integrins are not expressed on cardiac tissue but are present on circulating leukocytes (42). There are at least two mechanisms by which ICAM-1 and LFA-1 may contribute to the allograft rejection process, namely, by facilitating emigration of leukocytes into the cardiac tissue and providing the co-activation signals to the leukocytes (9, 11, 14–18). In the absence of co-activation signals, T cells may become anergic or even clonally deleted (44, 45). In addition to ICAM-1 and LFA-1, other adhesion molecules may facilitate emigration and provide co-stimulatory signals to leukocytes (9, 10, 42). In particular, three ligands (ICAM-1, ICAM-2, and ICAM-3) bind to LFA-1 and three ligands (LFA-1, Mac-1, and CD43) may bind to ICAM-1. Therefore, inhibition of ICAM-1 or LFA-1 alone may not be sufficient to produce a strong in vivo effect. In contrast, the combination of two agents that inhibit ICAM-1 and LFA-1 expression produced a potent immunosuppressive effect, thereby inducing tolerance.

In addition, IP-3082 synergistically prolonged heart allograft survival when combined with ALS, RAPA, or BQR. These immunosuppressive modalities act in different fashions: ALS decreases the level of T cells, including alloantigen-specific T cells (34); RAPA inhibits the transduction of signals delivered by lymphokines (35); and BQR blocks the dehydroorotate dehydrogenase enzyme that is required for pyrimidine synthesis (37). Interestingly, all three agents acting by distinct mechanisms interacted in a synergistic fashion with IP-3082 to prolong allograft survival, as documented by CI values below 0.02. In contrast, CsA, which blocks calcineurin activity, thereby inhibiting synthesis of lymphokines by T cells (38), did not potentiate the immunosuppressive effect of IP-3082. It is possible that CsA may inhibit ICAM-1 expression and that this might diminish the activity of IP-3082. However, CsA is not very effective in mice; as much as 75 mg/kg/day was required to block skin allograft rejection (46), and 20 mg/kg/day CsA was needed to fully protect vascularized heart allografts from rejection (Table V). In vivo treatment with 15 to 45 mg/kg/day CsA did not affect alloantigen-induced performances of explanted T cells (39). Because mice are resistant to the immunosuppressive effect of CsA, it is unclear whether the antagonism between CsA and IP-3082 is a result of a pharmacologic or a pharmacokinetic reason. Similarly, control IP-1082 combined with CsA did not affect heart allograft survival. However, CsA is very effective in rats (47): 2 mg/kg/day CsA prevented heart allografts from rejection, 10 mg/kg/day CsA delivered for 14 days induced indefinite allograft survival. These in vivo results correlated with a dose-dependent inhibition of in vitro immune responses by T cells obtained from immunosuppressed recipients. Therefore, we plan to evaluate the interaction between CsA and rat ICAM-1 antisense PS-oligo in rats.

ICAM-1 antisense IP-3082 seems to be well-tolerated at therapeutic doses without producing signs of toxicity. In fact, administration of IP-3082 to mice at a dose of 100 mg/kg/day q.o.d. for 14 days, did not produce any major toxic effects. Most important, in contrast with xenogeneic mAb, IP-3082 does not induce an antigenic response in animals (D. Kornbrust, unpublished observation). Furthermore, the pharmacokinetics of PS-oligos suggest that they are very efficient, and that they last long in vivo. Within 4 h, PS-oligos injected once i.v. accumulated predominantly in the liver (23%), kidney renal cortex (14%), medulla (3%), bone marrow (14%), and skin (13%). Metabolism of PS-oligos differed among organs. After 24 h, approximately 15% of the PS-oligo was intact in the liver, but as much as 40% was intact in the kidney (48). In blood, PS-oligos were found almost completely in the plasma fraction bound with low affinity to albumin and α_2-macroglobulin.

Experiments in monkeys were performed to examine the effectiveness of anti-ICAM-1 mAb (R6.5) to block rejection of kidney allografts (49). As in humans, ICAM-1 is expressed on the vascular endothelium in normal kidney. During rejection, ICAM-1 expression increased on endothelial and tubular cells and leukocytes, and this increase correlated with massive infiltration of grafts. Anti-ICAM-1 mAb treatment decreased cellular infiltration and allowed administration of lower CsA doses. Recent clinical trials conducted in high-risk kidney allograft patients showed that adding mouse anti-ICAM-1 mAb (BIRR1) in a 14-day postoperative period to the triple-drug therapy (CsA, azathioprine, and corticosteroids) improved 1-yr allograft survival from 56 to 78% (50). However, 16 of the 18 patients developed human anti-mouse Abs, which were detected between 3 to 14 days after completion of therapy with the BIRR1 mAb.

The PS-oligos used in this study for in vitro inhibition of ICAM-1 expression were added to the cultures in the presence of cationic liposomes. As previously shown, cationic liposomes enhance the activity of an ICAM-1 antisense PS-oligo (51–57). Interestingly, the same PS-oligo, IP-3082, that inhibited ICAM-1 expression in bEND.3 cells in the presence of cationic lipids, prolonged heart allograft survival when delivered to recipients by i.v. infusion suspended in saline without cationic liposomes. Similar observations have been noted with PS-oligos that targeted murine protein kinase C-α. An i.p. administration of this PS-oligo resulted in a selective reduction of protein kinase C-α mRNA in the livers of treated animals (58). Furthermore, several reports showed that local delivery of PS-oligos in the absence of cationic liposomes inhibited expression of the targeted gene (59–61). For example, a c-*myb* PS-oligo incorporated into a pluronic gel inhibited c-*myb* expression and smooth muscle cell proliferation in injured arteries (59). In another study, a c-*myb* antisense PS-oligo infused i.p. by a 7-day osmotic pump to *scid* mice bearing the human K562 myeloma leukemia tumor prolonged survival sixfold in comparison with untreated controls (62). Antisense, but not sense, PS-oligos complementary to the initiation codon of the nuclear factor-κB mRNA blocked development of fibrosarcoma in transgenic mice (63). To our knowledge, this study reports the first example of pharmacologic activity of an antisense PS-oligo by i.v. systemic administration in a model of a complex inflammatory process. In conclusion, our in vivo results demonstrate that antisense technology may proffer a new method of nontoxic and gene-targeted immunosuppressive treatment for organ transplantation.

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Disposition of the ^{14}C-Labeled Phosphorothioate Oligonucleotide ISIS 2105 after Intravenous Administration to Rats

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ABSTRACT

5′-TTGCTTCCATCTTCCTCGTC-3′ (ISIS 2105) is a phosphorothioate oligodeoxynucleotide currently being evaluated as an intralesional antiviral drug for the treatment of genital warts that are caused by the human papillomavirus. ISIS 2105, labeled with ^{14}C (at the carbon-2 position of thymine) was administered as a single i.v. injection (3.6 mg/kg) to female Sprague-Dawley rats to assess the disposition of the drug. After i.v. administration of [^{14}C]2105, blood radioactivity disappeared in a multiexponential manner with the half-lives of the phases equal to 0.4, 1.9, 7.1 and 5.1 hr. The initial volume of distribution was 22 ml and the postdistribution volume of distribution was 1076 ml, which indicated an extensive distribution of radioactivity. The apparent blood clearance was 14.7 ml/hr. The radioactivity in the expired air accounted for 51% of the administered dose over the 10-day period. Urinary and fecal radioactivity accounted for 15% and 5% of the administered dose, respectively. The major sites of radioactivity uptake were the liver (up to 22.6% of the dose), kidneys (renal cortex, up to 14% of the dose), bone marrow (up to 14% of the dose), skin (up to 13% of the dose) and skeletal muscle (up to 9% of the dose). Other tissues contained approximately 1% or less of the dose. The overall recovery of radioactivity 10 days postdosing was 95.1 ± 7.5% (mean ± S.D.) of the administered single dose. The radioactivity in the blood was almost completely in the plasma during the course of the study. In the plasma, the radioactivity was extensively bound to proteins, as assessed by size-exclusion high-performance liquid chromatography (HPLC), in samples up to 8 hr postdosing. Retention data on size-exclusion HPLC and *in vitro* incubations using purified proteins suggested that the plasma proteins that bound [^{14}C]2105 were albumin and α_2-macroglobulin. The complex formed between the plasma proteins and [^{14}C]2105-derived radioactivity was dissociated on anion-exchange HPLC to indicate that the great majority of plasma radioactivity coeluted with intact [^{14}C]2105 in samples that contained sufficient radioactivity for analysis. There was a time-dependent decrease in the proportion of hepatic and renal radioactivity that coeluted with the intact [^{14}C]2105 during the course of the study. The urine did not contain radioactivity that eluted with intact [^{14}C]2105 or anion-exchange HPLC.

Substantial interest in the development of oligonucleotide-based therapeutic agents has been generated (Zamencik *et al.*, 1978; Stein *et al.*, 1988; Mirabelli *et al.*, 1991; Crooke, 1992). Several first-generation oligonucleotide analogs, in which one or more of the substituents on the internucleotide phosphate are modified, *e.g.*, phosphorothioates, methylphosphonates and phosphorodithioates, have been synthesized and tested (Matsukura *et al.*, 1987; Crooke, 1992). Each of these modifications was shown to enhance the nuclease stability of oligonucleotides significantly (Agrawal and Goodchild, 1987; Crooke, 1991).

Phosphorothioate oligodeoxynucleotides have been studied extensively as potential antisense therapeutic agents. They displayed potent antiviral activities and inhibitory activities against a wide range of mammalian gene products (Mirabelli *et al.*, 1991; Crooke, 1992). Although phosphorothioate oligodeoxynucleotides may display pharmacological activities that are the result of mechanisms other than antisense, typically, non-antisense effects occur at doses significantly greater than the antisense effects (Mirabelli *et al.*, 1991; Crooke, 1992). Optimal antisense activities for phosphorothioate oligodeoxynucleotides are usually observed with oligonucleotides that are 18 to 21 nucleotides in length (Cohen, 1989).

Phosphorothioate oligodeoxynucleotides have been shown to be stable (half-lives > 24 hr) in serum, cell homogenates, cells, cerebrospinal fluid and organs (Crooke, 1991; Campbell *et al.*, 1990; Agrawal *et al.*, 1988; Crooke, 1993; Stein *et al.*, 1988; Loke *et al.*, 1989; Hoke *et al.*, 1991). They were taken up by many types of cells in tissue culture (for review, see Crooke, 1991; Crooke, 1993) and cellular uptake and *in vivo* activities can sometimes be enhanced by cationic lipids (Bennett *et al.*, 1992; Perlaky *et al.*, 1993).

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ABBREVIATIONS: HPLC, high-performance liquid chromatography; SE-HPLC, size-exclusion HPLC; SAX-HPLC, strong anion-exchange HPLC; ISIS 2105, 5′-TTGCTTCCATCTTCCTCGTC-3′; AUC, area under the plasma curve.

Two previous studies investigated the pharmacokinetics of phosphorothioate oligodeoxynucleotides in animals. The pharmacokinetics of a 20-mer phosphorothioate oligodeoxynucleotide were determined after single i.v. or i.p. doses of 30 mg/kg. The oligonucleotide was labeled at each internucleotide linkage with ^{35}S. The compound was shown to be bioavailable after i.p. administration to have broad peripheral tissue distribution and to be cleared primarily by renal excretion. Gel electrophoretic analysis demonstrated significant, albeit slow, metabolism in the liver, kidney and intestines (Agrawal *et al.*, 1991). A 27-mer phosphorothioate deoxyoligonucleotide labeled with ^{35}S was reported to display biexponential elimination in plasma with an elimination half-life in excess of 40 hr after a single i.v. dose of 4.8 mg/kg in the rat (Iversen, 1991).

ISIS 2105 is a phosphorothioate deoxyoligonucleotide that is active against human papillomaviruses (Cowsert *et al.*, 1993). It is currently undergoing pivotal Phase II clinical trials for the treatment of genital warts. There were two objectives of the current study. First, we wanted to develop and evaluate a radiolabeling method that results in higher-specific-activity oligonucleotides to support more detailed pharmacokinetic studies and a more definitive evaluation of metabolism that could be used in clinical trials. Second, we sought to perform more definitive pharmacokinetic distribution and metabolic studies in which metabolites in the plasma, urine and tissues were evaluated using HPLC techniques that support more quantitative analyses.

To achieve these objectives, we radiolabeled ISIS 2105 at the carbon-2 position of all thymidines. Because thymidine is metabolized into CO_2, metabolism can be measured by collecting expired air. This provides an estimate of the total metabolism of the oligonucleotide. Coupled with the extraction of radioactivity and HPLC analysis, a reasonably precise evaluation of metabolism can be achieved. Obviously, a full evaluation of metabolism will require the analysis of intermediates between the intact oligonucleotide and CO_2. We also developed HPLC methods to evaluate the integrity of the radiolabel in tissues and biological fluids.

Materials and Methods

ISIS 2105 Synthesis and Purification

The ^{14}C-labeled phosphorothioate ISIS 2105 was chemically synthesized by using the deoxynucleoside phosphoramidite approach (Beaucage and Caruthers, 1981; Matteucci and Caruthers, 1981). The phosphorothioate linkage was generated by oxidizing with ^{3}H-1,2-benzodithiole-3-one-1,1-dioxide (Iyer *et al.*, 1990) instead of aqueous iodine. All reagents and materials for the solid-phase synthesis of DNA were purchased from commercial sources with the exception of the ^{14}C-thymidine phosphoramidite. The ^{14}C-labeled phosphoramidite synthon was generated from ^{14}C-thymidine (^{14}C at the carbon-2 position of the thymine ring (specific activity, ~56.3 mCi/mmol, Sigma, St. Louis, MO) as described elsewhere (D. Dellinger and H. Sasmor, manuscript in preparation).

The crude synthetic oligonucleotide was purified by trityl-on reverse-phase HPLC by using a methanol gradient in a 0.25 M sodium acetate mobile phase buffer. The HPLC product was acid deprotected and recovered by ethanol precipitation as the sodium salt. The final product was analyzed by using electrophoresis with 20% denaturing polyacrylamide gels and the full length integrity (88% full length material) and the radiochemical purity (88% of counts per minute in the full length product) was determined by laser scanning densitometry and quantitative phosphorimaging (Molecular Dynamics, Foster City, CA), respectively. The specific activity of the final product was ~2.0×10^8 cpm/μmol and had approximately 97.6% phosphorothiodiester content (*vs.* 2.4% phosphodiester) as determined by high-field nuclear magnetic resonance (500 MHz, University of Missouri, Columbia, MO).

Formulation of ^{14}C-ISIS 2105 in phosphate-buffered saline. We formulated 2.22×10^9 cpm (11.7 μmol) of the purified product by dilution in phosphate-buffered saline, pH 7.0, (Irvine Scientific, Pasadena, CA) to deliver 2×10^7 cpm per 100-μl injection. The solution was sterile filtered through a 0.22-μm cellulose acetate filter (S&S Uniflow, Keene, NH) and the radioactivity was determined in~ 100 μl by aqueous scintillation.

Animals

Young adult female Sprague-Dawley rats (9–10 weeks old, 175–209 g at the time of randomization) were purchased from Taconic Farms (Germantown, NY). The animals were acclimated to the surroundings of the animal facility used for radioactive studies for approximately 1 week before dosing and were examined by a veterinarian before they were assigned to the study. During acclimation, the rats were housed in individual stainless steel suspended cages with noncontact bedding (Cellu-dri, Shepherd Specialty Papers, Kalamazoo, MI). Twenty-four hours before dosing, the rats to be used for urine, feces and expired air collection were transferred to Nalgene metabolism cages (Nalge, Rochester, NY). At the time of dosing, the animals used for the collection of urine, feces and expired air were transferred to glass metabolism cages (Vanguard International, Neptune, NJ). The animals used for the collection of retro-orbital blood samples, but not for urine and feces, were housed individually in stainless steel suspended cages. Each cage was labeled with the animal identification number. Food and water were allowed *ad libitum*. The food consisted of Purina Standard Rodent Chow (#5001, Raltech Scientific Services, St. Louis, MO) in pellet form. The water was city tap water.

Compound Administration

The [^{14}C]2105 was administered in solution in phosphate buffer, pH 7, to rats by i.v. injection (100 μl) into the caudal tail vein. The concentration of [^{14}C]2105 was 1.2 mM; therefore, the dose level was approximately 3.6 mg/kg. The actual doses administered were calculated by using the assayed concentration of radioactivity in a 100-μl volume of the formulation.The [^{14}C]2105 was administered to 25 female rats. Five rats were used for the collection of urine, feces, expired air and tissues at the time of sacrifice. The remaining 20 animals were used for the collection of blood at intervals after dosing and tissues at the time of sacrifice.

Sample Collection

Urine and feces. Urine and feces were collected from the five animals housed in glass metabolism cages at 0 to 4, 4 to 8, 8 to 24, 24 to 48, 48 to 72, 72 to 96, 96 to 120, 120 to 144, 144 to 168, 168 to 192, 192 to 216 and 216 to 240 hr after administration of [^{14}C]2105.

The urine and feces were frozen immediately on excretion and were kept frozen for the entire collection period. Before sacrifice, the animals were made to urinate by gentle pressure on the urinary bladder and this urine was combined with the last sample collected. A thorough cage wash with water was performed at the time of sacrifice. At sacrifice, the blood and plasma were obtained as described subsequently. Urine, feces and cage washings were stored in a −20°C freezer.

Expired air. The radioactivity in the expired air was collected from the five animals housed in glass metabolism cages in a series of two traps. The traps contained 6 M KOH for the collection of $^{14}CO_2$. The radioactivity in the expired air was collected during the following intervals: 0 to 4, 4 to 8, 8 to 24, 24 to 48, 48 to 72, 72 to 96, 96 to 120, 120 to 144, 144 to 168, 168 to 192, 192 to 216 and 216 to 240 hr after the administration of [^{14}C]2105. The KOH samples were stored in a 4°C refrigerator until analysis.

Blood. Single blood samples (approximately 50–100 μl each) were obtained by retro-orbital puncture from two animals at each of the following time points: 0.5, 1.5, 3, 6, 12, 36, 56, 80, 104 and 128 hr. Capillary tubes containing blood were stored on ice until the blood was

aliquoted into combustion cones. Two animals were sacrificed at the following time points: 1, 2, 4, 8, 24, 48, 72, 96, 120 and 144 hr after dosing. At sacrifice, the animals were anesthetized with CO_2 and the blood (5 ml) was recovered by cardiac puncture and transferred into heparinized Vacutainer tubes (Becton Dickinson, Rutherford, NJ). The plasma was obtained from the blood by centrifugation and kept on ice before storage at −20°C. Animals were sacrificed by exsanguination.

Tissue. At the time of sacrifice, the liver, kidneys, spleen, lungs, brain, eyes, bone (femur), skeletal muscle (representative sample), ovaries, uterus, representative dorsal skin and carcass were collected and immediately frozen on dry ice and stored in a −20°C freezer.

Analytical Procedures

All determinations of total radioactivity in the tissues, excreta and blood were made with a Beckman LS 6000 scintillation system (Fullerton, CA).

The weight of the urine was determined. The radioactivity in duplicate samples of urine, plasma and cage rinse was determined in Scint-A XF (Packard Instrument, Downers Grove, IL). The radioactivity in duplicate samples of KOH was determined in modified Bray's solution (Nomeir *et al.*, 1992). Feces were weighed and homogenized with distilled water (20% w/w) with a Brinkmann Polytron homogenizer (Lucerne, Switzerland).

The liver, lung, brain and spleen samples were weighed and finely minced before combustion. The skeletal muscle (representative sample), blood, uterus, eyes and ovaries were weighed but not pretreated before combustion. The bone marrow was removed from bone and weighed before combustion. The renal medulla and cortex were excised from the kidneys and aliquots were weighed before combustion. The carcasses from the animals that were used to determine mass balance and the skin (representative sample) were weighed, powdered on dry ice with a Waring (New Hartford, CT) blender and homogenized with distilled water (33% w/w) with a Brinkmann Polytron homogenizer.

Duplicate samples (total sample size allowing) of fecal homogenates, tissue minces and whole tissues were aliquoted and then underwent combustion with a Packard Tricarb oxidizer, model 307. The ^{14}C radioactivity was trapped in Carbosorb II (Packard Instrument). The recovery of the combustion system was determined on a daily basis and ranged from 97% to 100%.

Extraction of tissue radioactivity. To approximately 1 g of liver, 0.5 ml of extraction buffer (0.5% NP-40, 20 mM Tris HCl, pH 8.0, 20 mM EDTA, 100 mM NaCl and 2 mg/ml of proteinase K) was added and the tissue was homogenized using a Bessman tissue pulverizer (Spectrum, Houston, TX) followed by using the A pestle of a 7-ml Dounce-type tissue grinder (Wheaton, Millville, NJ). One kidney from each animal was dissected into the cortex and medullar regions. We combined 0.1 to 0.2 g with 0.25 ml of extraction buffer and homogenized it in a 1-ml Dounce-type tissue grinder (Wheaton) by using the A pestle. All samples were then incubated at 65°C for 24 hr, followed by centrifugation at 16,000 × *g* at 4°C for 15 min. The supernatants were removed and stored frozen at −70°C until analysis.

All samples were filtered through an Ultrafree-MC 0.22-μm filter (Millipore, Bedford, MA) at 4°C. Before analysis, the liver and kidney samples were thawed and unlabeled ISIS 2105 was added to a final concentration of 100 μM. The plasma samples were thawed at room temperature and immediately analyzed.

HPLC. SAX-HPLC was used to determine the metabolic profile of the plasma and urine and hepatic and renal tissues. Ion-exchange analyses were carried out using a Beckman System Gold liquid chromatography system with model 126 pumps, model 507 autoinjector, and model 166 detector. We analyzed 40 μl of each sample of plasma and urine, or of liver or kidney homogenate, at 260 nm on a 4.6 × 100-mm Gen Pak Fax column (Waters, Milford, MA) by using the following buffers and gradient: buffer A, 0.086 M Tris HCl, pH 8.0, 20% methanol; buffer B, 0.086 M Tris HCl, pH 8.0, 1.5 M NaBR; gradient, 0% B isocratic for 5 min and then linear to 60% B over 45 min at a flow of 0.5 ml/min. Fractions (0.5 ml) were collected and added to 5 ml of Readysafe scintillation cocktail (Beckman) and then counted in a Beckman model LS6000IC scintillation counter.

The plasma samples were also analyzed by SE-HPLC by using a Hewlett-Packard (Pasadena, CA) model 1090 M liquid chromatography system and a 7.8 × 300-mm TSK-gel G2000 SWXL column (Tosohaas, Montgomeryville, PA). The analyses were carried out in 0.05 M Na_2HPO_4, 0.1 M Na_2SO_4 and 0.05 M NaH_2PO_4, pH 7.0, at a flow of 0.45 ml/min. On-line radiochemical detection was accomplished with a Radiomatic FLO-ONE/beta model A-525A detector (Packard Instruments, Meriden, CT). The scintillation cocktail, Ultimate-Flo-V, was purchased from Packard Instruments and was used at a flow rate of 0.9 ml/min.

Calculations

The concentrations of radioactivity in the samples processed by combustion were corrected for the recovery efficiency of the combustion system, which was determined daily before the combustion of experimental samples. The observed radioactivity values were converted to compound radioequivalent concentrations. The radioequivalents were defined as the amount of parent compound, at the specific activity as administered, that would result in the observed disintegrations per minute value. Compound equivalents in a biological sample were determined by dividing the disintegrations per minute in the sample by the specific activity of the compound in disintegrations per minute per microgram. The compound equivalents were expressed in micrograms per gram of tissue and, when possible, as a percentage of the administered dose/organ or tissue. For the purpose of calculating a mean ± S.D., the tissue samples in which the radioactivity was less than twice the background for the system, the equivalents were less than 0.005 microgram equivalents per gram or the radioactivity was less than 0.005% of the dose were considered to have a value of zero.

The radioactivity in urine, feces, volatile traps and cage rinse was expressed as a percentage of the administered dose for each time interval and as a cumulative percentage. For the purposes of calculating a mean ± S.D., urine, feces, expired air and cage wash samples in which the radioactivity was less than twice the background for the system or the radioactivity was less than 0.05% of the dose were considered to have a value of zero.

Pharmacokinetic parameters for the ISIS 2105 equivalents in blood and plasma were calculated by polyexponential curve fitting of the observed concentrations, using the RSTRIP, Polyexponential Curve Fitting Program, Version 4.02 (Micromath Scientific Software, Salt Lake City, UT). The areas under the concentration-time curve and the terminal elimination half-lives for tissues were calculated by using noncompartmental analysis of the observed data (Shumaker, 1986).

Results

Percentage of the Dose of [^{14}C]2105 in Tissues

The percentage of the dose in the tissues at intervals after the single-dose administration is summarized in table 1. The maximal percentage of the dose in the liver (23%) was observed 4 hr after dosing. The maximal percentages in the renal cortex (14%) and renal medulla (3%) were observed at 4 and 8 hr after dosing, respectively. At 240 hr after a single i.v. administration, a significant portion of the radioactivity remained in the tissues. The highest percentage of the dose was observed in the renal cortex (4%), followed by the skin and bone marrow (3% each). A lesser percentage was observed in the liver (2%). All other tissues contained less than 1% of the dose. The total percentage of the dose recovered in the tissues and carcass at 240 hr was approximately 20% (table 2).

Urinary Excretion of [^{14}C]2105-Derived Radioactivity

The mean cumulative percent of [^{14}C]2105-derived radioactivity excreted in the urine is summarized in figure 1. The

TABLE 1

Percentage of dose in tissues[a] of female Sprague-Dawley rats at intervals after a single i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg

Time	Liver	Renal Medulla[b]	Renal Cortex[b]	Lung	Skeletal Muscle[c]	Bone Marrow[d]	Skin[c]
hr				*Fraction of dose (%)*			
1	16	1	7	1	9	7	9
2	20	1	10	—[f]	7	8	13
4	23	2	14	—	5	10	12
8	19	3	12	—	2	12	8
14	14	1	13	—	1	14	9
48	10	2	10	—	2	13	2
72	8	2	10	—	—	10	11
96	6	1	8	—	—	8	8
120	4	1	7	—	—	8	4
144	3	1	6	—	—	5	6
240[e]	2	—	4	—	—	3	3

[a] Tissues containing ≥ 1% dose are included. Brain, spleen, ovaries, uterus and eyes contained < 1% dose at any given time.
[b] The percentages were calculated by assuming that renal cortex = 69% and renal medulla = 31% of the total kidney weight (the percentages were generated from kidney dissections in ADL MAP laboratory).
[c] The percentages were calculated from the organ weights and by assuming that muscle = 50% of the body weight and skin = 11% of the body weight (Burka *et al.*, 1987).
[d] The percentages were calculated by assuming that bone marrow = 3% of the total body weight (Baker *et al.*, 1979).
[e] All percentages are average data from two animals except for time 240 hr, which is the mean data from five animals.
[f] Contained < 1% of dose.

TABLE 2

Recovery of drug-related radioactivity from female Sprague-Dawley rats 240 hr after a single i.v. administration of [^{14}C]2105 at a dose of approximately 3.6 mg/kg

Sample	Animal No.					Mean ± S.D.
	2921	2922	2923	2924	2925	
			Dose recovered (%)			
Urine	17.5	16.2	15.3	15.0	11.5	15.1 ± 2.2
Feces	4.5	4.2	4.3	5.9	4.1	4.6 ± 0.7
Expired air	51.8	51.6	58.1	50.4	43.7	51.1 ± 5.1
Tissues	18.1	21.6	18.3	22.9	20.5	20.3 ± 2.2
Cage wash	3.8	4.0	4.8	5.0	2.3	4.0 ± 1.1
Total	95.7	97.6	100.8	99.2	82.1	95.1 ± 7.5

percentage of [^{14}C]2105-derived radioactivity excreted by the urinary route was 15.1 ± 2.2%, primarily within the first 72 hr. The urinary excretion rate resulted in an elimination half-life of 55 hr (table 3).

Fecal Excretion of [^{14}C]2105-Derived Radioactivity

The mean cumulative percent of [^{14}C]2105-derived radioactivity eliminated in the feces is summarized in figure 1. The percentage of [^{14}C]2105-derived radioactivity excreted in the feces was 4.6 ± 0.7%, primarily within the first 96 hr.

Excretion of [^{14}C]2105-Derived Radioactivity in Expired Air

The excretion of [^{14}C]2105-derived radioactivity in expired air is illustrated in figure 1. The majority of the i.v. dose of [^{14}C]2105 (51.1 ± 5.1% of the dose) was eliminated by expired air, primarily within the first 96 hr. The expiration rate of [^{14}C] 2105-derived radioactivity resulted in an elimination half-life of 60 hr (table 3).

Total Recovery of [^{14}C]2105-Derived Radioactivity

The total recovery of [^{14}C]2105-derived radioactivity after a single i.v. dose of [^{14}C]2105 at 3.6 mg/kg is summarized in table 2. The majority (51%) was recovered in the expired air. A smaller percentage was recovered in the urine (15%) and in the feces (4.6%). The remaining radioactivity was recovered in the tissues and carcass (20%) and the cage wash (4%). Overall recoveries ranged from 82% to 101% with a mean for the five animals of 95.1 ± 7.5%.

Pharmacokinetics of ISIS 2105 Equivalents in Blood and Tissues

The pharmacokinetic data analysis is based on microgram equivalents of ISIS 2105 present in the matrices. As such, the data describe the pharmacokinetic behavior of ISIS 2105-related radioactivity and not necessarily unchanged parent ISIS 2105.

Pharmacokinetics in blood. After i.v. administration, a peak blood radioactivity concentration of 17.2 μg equivalents/g was achieved. The concentration *versus* time profile in the blood is shown in figure 2, along with the fitted polyexponential curve used to calculate pharmacokinetic parameters. The blood radioactivity *versus* time profile was polyexponential, with four phases (table 3). The initial phase had a half-life of 0.4 hr and the terminal elimination phase had a half-life of 51 hr. The plasma data paralleled the blood elimination profile but the terminal elimination half-life was 40 hr (data not shown). At each of the corresponding time points, all or most of the radioactivity was associated with plasma and not with formed elements of blood. These data strongly suggest no binding or distribution of ISIS 2105 on or in the red blood cells. As would be expected from the long terminal half-life, the apparent blood clearance after i.v. administration was low, *i.e.*, 14.7 ml/hr (table 3).

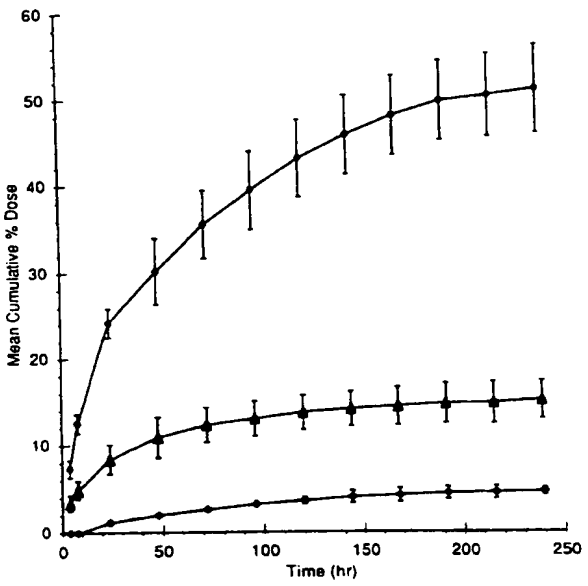

Fig. 1. Mean (± S.D., $n = 4$) cumulative percentage of the dose eliminated as $^{14}CO_2$ (●), in urine (▲) and in feces (◆) of female Sprague-Dawley rats after i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg.

The initial volume of distribution was 22.0 ml and the post-distribution volume of distribution was 1076 ml, which indicates extensive partitioning into the tissues.

Pharmacokinetics of tissue radioactivity. The tissue radioactivity concentrations were highest in the liver, kidney, spleen and bone marrow (table 1). The elimination half-life from the liver was 62 hr, which approximated that seen in the blood. The elimination half-life was prolonged in the kidney, with observed values of 112 hr in the cortex and 156 hr in the medulla. The elimination half-life of radioactivity from the bone marrow was 78 hr (table 3).

The areas under the concentration *versus* time curves for the tissues (bone marrow, liver, spleen and kidney) were calculated to gauge a relative drug exposure level after i.v. administration of [^{14}C]2105. For these four tissues, comparisons were made per gram of tissue and not adjusted for the total organ weights. Of these tissues, the liver and spleen received the lowest exposure, with $AUC_{(0-\infty)}$s of 1158 and 1618 μg equivalents-hr/g, respectively. The kidney medulla received 5.6 times the liver exposure or 6497 μg equivalents-hr/g. The kidney cortex received the highest exposure, 15688 μg equivalents-hr/g, approximately 2.4 times the medulla and 13.5 times the liver exposure. The bone marrow exposure was approximately 2 times the liver exposure, with an AUC of 2526 μg equivalents-hr/g. These AUCs were much higher than the blood AUC; this again suggested significant partitioning of ISIS 2105 equivalents into the tissues.

Characterization of Plasma, Tissue and Urinary Radioactivity

Plasma radioactivity was present in two peaks as assessed by SE-HPLC (fig. 3). The majority of the radioactivity eluted with a retention time that was the same as that of the known complex formed between purified rat albumin and [^{14}C]2105 (approximately 18 min). A smaller proportion of the plasma radioactivity eluted with a retention time that was the same as that of the known complex formed between purified human α_2-macroglobulin and [^{14}C]2105 (approximately, 15 min). Little or no radioactivity eluted with a retention time of authentic [^{14}C] 2105 (approximately 23 min). Preliminary studies (Cossum *et al.*, manuscript in preparation) demonstrated that the affinity of ISIS 2105 for albumin and α_2-macroglobulin was in the micromolar range and that binding to both proteins was saturated when ISIS 2105 concentrations exceeded 5 to 10 μM.

To determine the integrity of ISIS 2105, plasma was applied directly to SAX-HPLC. The buffer in that system results in the extraction of radioactivity from plasma proteins. When a tissue pulverizer step was included, the total recovery of the radioactivity was approximately 60%. If only simple homogenization with a Dounce homogenizer was used, only approximately 30% of the total radioactivity was recovered. However, a comparison of the samples by HPLC revealed no differences; thus, both methods probably extract representative samples from the tissues. When plasma sampled from rats for up to 8 hr postdosing was subjected to SAX-HPLC, the majority of the radioactivity eluted with authentic [^{14}C]2105 (fig. 4). The radioactivity in peaks eluting earlier than authentic [^{14}C]2105 were, presumably, shorter metabolites of [^{14}C]2105. At the 8-hr time point, approximately 38% of the radioactivity represented intact ISIS 2105. A determination of the proportion of intact ISIS 2105 in the plasma obtained after 8 hr (*i.e.*, ≥ 24 hr) was not possible because of the low levels of radioactivity in those samples.

Figures 5 and 6 show anion-exchange radiochromatograms of extracts of hepatic and renal cortex tissues, respectively,

TABLE 3
Pharmacokinetic parameters of ISIS 2105 equivalents after a single i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg

Sample	AUC	Distribution $T_{1/2}$	Terminal $T_{1/2}$	C_{max}	T_{max}	Apparent Clearance	V_c[a]	V_{dss}[b]
	μg equivalents-hr/g	hr	hr	μg equivalents/g	hr	ml/hr	ml	ml
Blood	52	7.1	51	17.2	0.5	14.7	22.0	1076
Urine	—		55	—	—			
Expired air	—		60	—	—			
Liver	1158		62	19.0	4			
Renal medulla	6497		156	42.7	8			
Renal cortex	15,688		112	87.4	4			
Spleen	1618		163	10.6	1			
Bone marrow	2526		78	18.1	24			

[a] Initial volume of distribution.
[b] Postdistribution volume of distribution.

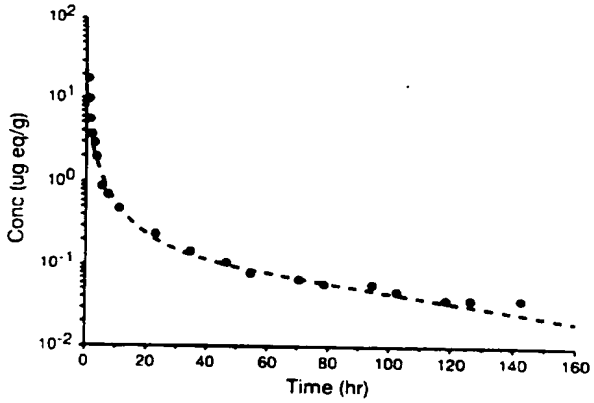

Fig. 2. Concentration of ISIS 2105 equivalents in the blood of female Sprague-Dawley rats after i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg. Each point represents the average of the values for two animals. The dashed line represents the line of best fit estimated from the four-compartment model.

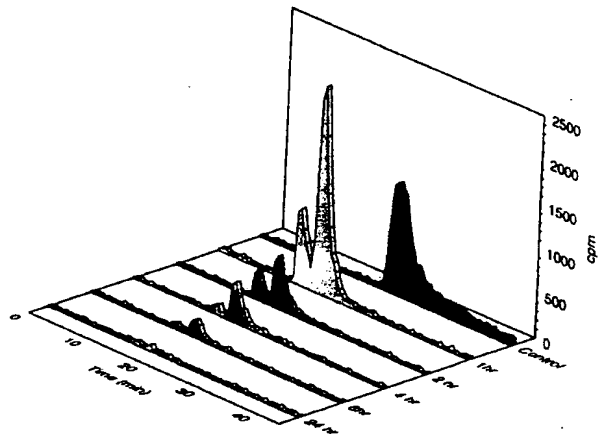

Fig. 3. SE-HPLC radiochromatograms of plasma sampled from female Sprague-Dawley rats at various times after i.v. administration of [^{14}C] 2105 at a dose of 3.6 mg/kg. The control peak represents [^{14}C]2105 that has a retention time of approximately 23 min. The plasma samples from dosed rats contained radioactive peaks eluting at approximately 15 min and approximately 18 min.

sampled at various times after the administration of [^{14}C]2105. The proportion of apparently unchanged [^{14}C]2105 decreased with time. The proportion of radioactivity present as apparently intact [^{14}C]2105 varied between the liver and kidney; [^{14}C]2105 was more stable in the kidney than in the liver. After 24 hr, approximately 15% of the radioactivity extracted from the liver represented intact ISIS 2105 and only trace levels of intact ISIS 2105 were present at 48 hr. By contrast, even after 96 hr, 48% of the radioactivity extracted from the kidney represented intact ISIS 2105.

Figure 7 shows anion-exchange radiochromatograms of urine sampled for up to 96 hr postdosing. Little or no radioactivity eluted with the same retention time as authentic [^{14}C]2105 at any sampling time.

Discussion

The radioactivity in the blood was located almost entirely in the plasma for 3 to 4 days after dosing. Only low levels of radiolabel were detected associated with the cellular components of the blood. The radioactivity in the plasma was associated with albumin and α_2-macroglobulin; only a trace of free [^{14}C]2105 was detectable in the plasma. Preliminary data suggest that ISIS 2105 binds to these proteins with a relatively low affinity and that binding is clearly saturable (Cossum *et al.*, manuscript in preparation). The radioactivity bound to those plasma proteins represented mostly intact [^{14}C]2105. However, the levels of radioactivity in plasma were insufficient to determine the integrity of the drug at times beyond 8 hr postdosing.

There was a rapid and substantial distribution of radioactivity from the blood into the tissues. The initial volume of distribution of 22.0 ml approximates the blood volume of the rats used in this study. The postdistribution volume of distribution was 1076 ml, a value that indicated the distribution of radioactivity into a "deep" compartment. A four-compartment model fit the data best (*i.e.*, r^2 0.998 *vs.* 0.823 for a two-compartment model). However, in other studies we have performed, a two-compartment model fit the data best. Consequently, we think the complexity of the model most likely results from minor animal-to-animal variations and the fact that radioequivalents were considered rather than intact drug. In future studies, we will attempt to address this issue in more detail. In any event, the elimination half-life was prolonged and examination of intact drug levels suggested a relatively prolonged elimination half-life for the intact drug and radioequivalents. The primary organs of accumulation of radioactivity were the liver, kidneys (particularly the renal cortex), bone marrow and spleen. The kinetics of distribution of the radiolabel into the peripheral organs varied. Peak levels were achieved in the liver and kidney 4 hr after the dose. By contrast, peak levels in the skeletal muscle were observed 1 hr after the dose and peak levels in the bone marrow were not achieved until 24 hr postdose. Skin accumulated a surprising amount of radiolabel, with peak levels that occurred 2 to 4 hours after administration.

That the radioactivity in various organs represented intact [^{14}C]2105 and metabolites was demonstrated by extraction followed by SAX-HPLC. Although its metabolism in the liver was extensive, the rate of metabolism was relatively slow. Twenty-four hours postdosing, approximately 15% of the total hepatic radioactivity was present as intact [^{14}C]2105. The metabolism in the kidney was minimum because intact drug was present even 96 hr postdosing. The extensive metabolism in the liver, coupled with the lack of metabolites found in the kidney and complete absence of intact drug in the urine, suggested that, after the initial distribution, only limited redistribution between the liver and kidney (and presumably other organs) occurred.

In this study, ^{14}C-labeled ISIS 2105 was synthesized using ^{14}C-labeled thymidine labeled at the carbon-2 position of thymine. The fate of thymidine is either through utilization into DNA or degradation to thymine (Henderson and Patterson, 1973). In mammals, the carbon-2 position carbon of thymine is degraded to CO_2 and so production of $^{14}CO_2$ would be expected when thymine is labeled at that carbon (Henderson and Patterson, 1973). Thymine could have been generated from ISIS 2105 *in vivo* in at least two ways. The putative metabolic scheme would involve hydrolysis of the phosphorothioate backbone, which eventually would generate thymidine. Subsequently, thymidine phosphorylase could dethiophosphorylate the thymi-

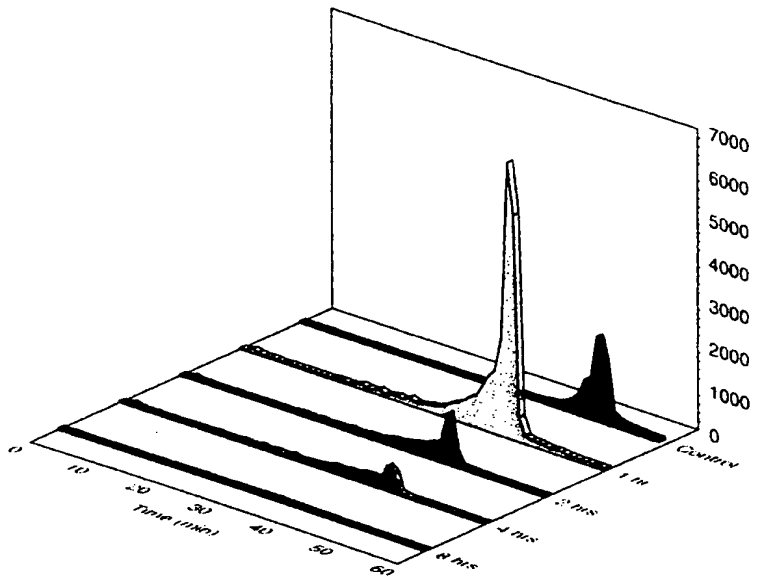

Fig. 4. SAX-HPLC radiochromatograms of plasma sampled from female Sprague-Dawley rats at various times after i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg. The plasma contained radioactivity that eluted principally with [^{14}C]2105 (approximately 43 min).

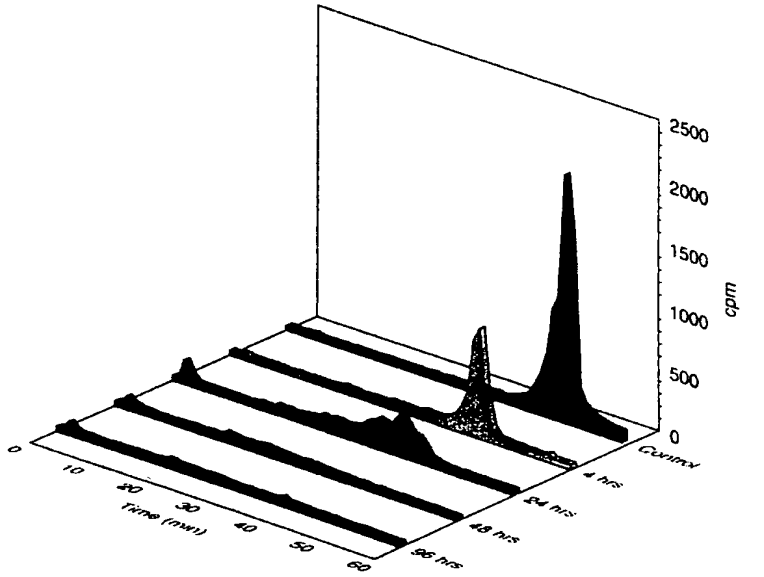

Fig. 5. SAX-HPLC radiochromatograms of extracts of liver sampled from female Sprague-Dawley rats at various times after i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg. Authentic [^{14}C]2105 elutes at approximately 43 min.

dine or the thiophosphate might be oxidized first and the phosphorylase then would release phosphate. Thymine could then be further metabolized to CO_2. Thymidine phosphorylase is a cytoplasmic enzyme with highest activity in the intestinal mucosa, liver, bone marrow, kidney and spleen (Friedkin and Roberts, 1954). Consequently, the limited amounts of degradation products in the kidney suggest that the rate-limiting step in the pathway is hydrolysis. This is consistent also with the slow overall metabolism observed. In this regard, it is important also to recognize that approximately 2.4% of the internucleotide linkages in [^{14}C]2105 were phosphate resulting from the oxidation of the phosphorothioate during synthesis. This is greater than the routine specification for unlabeled ISIS 2105 (0.6%) and could account for a slightly greater hydrolytic rate of radiolabeled ISIS 2105.

Alternatively, it is possible that thymine was removed from [^{14}C]2105 by a glycosidic bond cleavage without prior hydrolysis of the internucleotide linkage. DNA glycosylases are nuclear enzymes that remove purines or pyrimidines from DNA as part of repair mechanisms. The resulting apurinic or apyrimidinic site in the oligonucleotide would be expected to be susceptible to endonuclease action (Warner, 1983). Further studies are required to elucidate the mechanism(s) of ISIS 2105 degradation.

The principal, albeit slow, mechanism of clearance of ISIS 2105 in rats is metabolism. Of the total dose, in excess of 50%

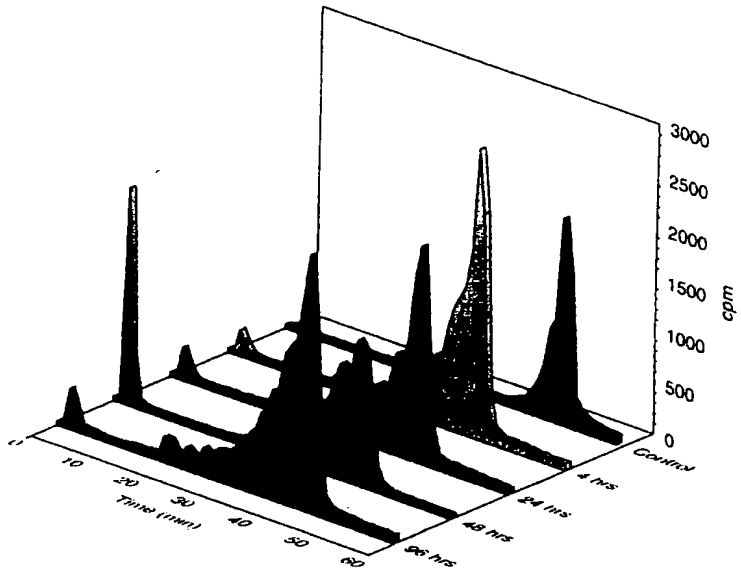

Fig. 6. SAX-HPLC radiochromatograms of extracts of renal cortex sampled from female Sprague-Dawley rats at various times after i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg. Authentic [^{14}C]2105 elutes at approximately 43 min.

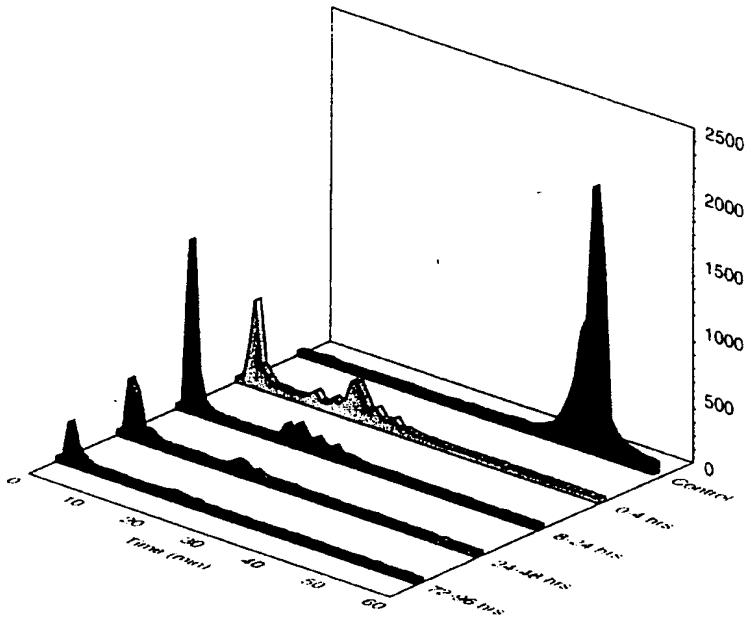

Fig. 7. SAX-HPLC radiochromatograms of urine sampled from female Sprague-Dawley rats at various times after i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg. Authentic [^{14}C]2105 elutes at approximately 43 min.

was recovered in expired air. Only metabolites were found in the urine and urinary excretion accounted for only 15% of the total dose. There was no evidence of significant biliary secretion or hepatobiliary recirculation.

Although the results of this study and the study of Agrawal *et al.* (1991) were similar, there were several important differences. Agrawal *et al.* (1991) did not report on plasma protein binding. ISIS 2105, and every other phosphorothioate oligonucleotide we have studied, binds extensively to plasma proteins. The binding is low affinity and high capacity, which is traditionally associated with many other classes of drugs and their interaction with plasma proteins. We consider this to be one of the principal reasons that phosphorothioate oligonucleotides are not cleared rapidly by renal filtration. At the 30-mg/kg dose used by Agrawal *et al.* (1991), we would expect the plasma protein binding to be saturated and, therefore, to result in significant levels of free drug in the plasma. Approximately 30% of the i.v. or i.p. dose of radioactivity after dosing with the ^{35}S-labeled oligonucleotide was recovered in the urine by 24 hr postdosing and gel electrophoresis of the urine indicated the presence of intact drug. At early times (0–6 hr) after i.v. dosing, 95% of the urinary radioactivity coeluted with intact drug and,

up to 24 hr postdosing, only 15% degradation was noted. The urine of mice dosed i.p. contained material that was only 10% intact parent drug at 24 hr postdosing. At no time did we observe intact drug in the urine.

Two preliminary reports of studies on an anti-*rev* 27-mer phosphorothioate oligodeoxynucleotide have been presented (Bigelow *et al.*, 1991; Bigelow *et al.*, 1992). In these studies, the drug was given by a variety of routes (i.v. bolus, i.v. infusion, s.c. and p.o.) and concentrations of intact drug were determined by HPLC separation and ultraviolet detection. Excellent bioavailability from s.c. sites and limited p.o. bioavailability were reported. Tissue accumulation similar to our results was reported. A significantly shorter plasma half-life was reported but this was probably the result of the relatively insensitive detection methods. We have studied the pharmacokinetics of several oligonucleotides after intradermal, i.m., i.p. and intravitreal administration and articles describing these results are in preparation.

Agrawal *et al.* (1991) reported that 85% to 90% of the radioactivity present in most tissues of mice 48 hr after the administration of a ^{35}S-labeled phosphorothioate oligonucleotide was associated with intact drug. However, only 50% of the radioactivity in the liver and kidneys was associated with the parent drug at 48 hr. Only approximately 5% of hepatic radioactivity was present as apparently unchanged [^{14}C]2105. By contrast, the majority of renal cortex radioactivity in 96-hr samples eluted with authentic [^{14}C]2105. Larger molecular weight bands were found when tissue extracts were analyzed by polyacrylamide gel electrophoresis in the study of Agrawal *et al.* (1991). They speculated that the radioactivity might represent longer oligonucleotides. We did not observe any evidence of these species. An alternative explanation for these observations might be that the material represented drug bound to α_2-macroglobulin, a protein which is found in mice (LaMarre *et al.*, 1991).

All these differences might be explained by variations between species or differences caused by different sequences. Moreover, Iverson (1991) reported that, at lower doses, an anti-*rev* oligonucleotide was excreted intact in urine, which further suggests that there may be sequence differences. However, in studies in our laboratories, we have not observed such significant differences as a function of species or the specific sequence of the drug. Additional studies are clearly indicated.

In summary, after i.v. administration of [^{14}C]2105 to rats, the tissue distribution of radioactivity was extensive and the radioactivity was eliminated slowly. Although the drug was apparently extensively metabolized, the available evidence shows that the rate of metabolism was relatively slow. Metabolic studies were facilitated by synthesizing an oligonucleotide containing ^{14}C-labeled thymidine. Circulating radioactivity was extensively bound to plasma proteins, a phenomenon that may retard the renal filtration of unchanged drug. Finally, we observed binding of ISIS 2105 to α_2-macroglobulin. In addition to providing a plasma reservoir of ISIS 2105, binding to α_2-macroglobulin could be important in the pharmacokinetics of ISIS 2105 because this protein has been shown to be taken up by various cells through a receptor-mediated mechanism (James, 1990).

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